Identifizierung und Charakterisierung von Metallchelat-bindenden Peptiden mittels Phage-Display

Von der Gemeinsamen Naturwissenschaftlichen Fakultät

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D i s s e r t a t i o n

von Jörn Felix Glökler

aus Tübingen

1. Referent: Prof. Dr. John Collins

2. Referent: Prof. Dr. Singh Chhatwal

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1 INTRODUCTION

1.1 Phage-Display

1.1.1 Combinatorial libraries

During recent years, novel combinatorial techniques have been developed to select individual interacting partners from an enormous diversity of molecules. In general, two approaches can be discriminated. The so-called "rational" design, which relies on data provided by the constantly growing number of already identified interactions, and the "irrational" approach by the empirical screening for possible binding partners. The former is conducted *in silicio* using computer aided design and faces severe limitations when results are to be reproduced *in vitro* due to the complexity of parameters involved. The latter is mainly limited by the diversity of the combinatorial library screened which represents the so-called "sequence-space". The greater the diversity of the library, the more likely it will contain an avidly binding molecule displaying the desired properties. Such combinatorial libraries can either be composed of synthetic molecules or consist of replicating organisms such as viruses or cells. Examples are the oriented synthetic peptide libraries, the yeast two-hybrid system, a novel bacterial two-hybrid system and bacterial surface display (Frank, 1992; Allen *et al*., 1995; Karimova *et al*., 1998; Stahl and Uhlen, 1997). Exceptions are aptamer and ribosomal-display libraries (Gold *et al.*, 1995; Hanes and Plückthun, 1997), allowing both screening and amplication *in vitro*. For the identification of variants in synthetic libraries, a sufficient number of molecules have to be recovered from the screening process. This limits the feasibility of such a library to a diversity up to 10^8 individual variants in one ml.

In order to cover a larger "sequence space", phage-display offers the most powerful option. This technique was initially introduced in 1985 by G.P. Smith (Smith, 1985). It employs the use of filamentous M13-like *Eschericha coli* F^+ strain infecting bacteriophage. The advantage over the synthetic libraries is the physical coupling of phenotype and genotype. This enables the identification of a **single** binding molecule, displayed as protein or peptide fused to the surface of a bacteriophage by sequencing the encoding genome after amplification. Up to 10^{14} M13-like bacteriophage can be contained in one ml. Therefore, library size is primarily limited by the efficiency of transformation of *E. coli* enabling realistic library sizes up to 10^{11} different variants (Collins, 1997).

1.1.2 Filamentous bacteriophage

Filamentous bacteriophage (M13, fd, f1, IKe) of *E. coli* possess a circular, covalently closed single-stranded DNA (ssDNA), surrounded by a cylinder of coat. The genome consists of 9 genes encoding 11 proteins (pI-pXI). Two of these proteins, pX and pXI, are products of internal translational initiation of gene II and III, respectively (Model and Russel, 1988). The minor coat protein pIII of filamentous bacteriophage is essential for infectivity. It possesses a tripartite structure, in which single domains are separated by glycine-rich linkers. Crystal structures of the first domains D1 and D2 have been determined (Lubkowski *et al*., 1998; Holliger *et al*., 1999), demonstrating a horseshoe-like conformation of the two structurally related domains. The C-terminal domain D3 is known to be required for pIII incorporation into the phage particle and release from the inner membrane (Stengele *et al*., 1990; Rakonjac *et al*., 1999). Filamentous phage infect *E. coli* by binding of D2 to the tip of a sex-pilus encoded by the F episome in male strains. As the pilus retracts to the cell surface, D1 binds to the C-terminal domain (TolAIII) of the TolA protein, a subunit of the TolQRA pore-complex present in the periplasm (Derouiche *et al*., 1996). Interestingly, pIII shares similarities with colicins such as colicin Ia in terms of structure and uptake mechanism (Derouiche *et al*., 1997; Riechmann and Holliger, 1997; Click and Webster, 1997; Raggett *et al*., 1998). By transferring the major coat protein into the inner membrane at the TolQRA complex, the phage genome is released into the cytoplasm of the cell (Click and Webster, 1998). The invading ssDNA is replicated to many copies of the double stranded replicative form (RF) by involvement of pII. In the meantime, the remaining phage genes are transcribed and translated. The coat proteins (pIII, pVI, pVII, pVIII and pIX) of the progeny phage accumulate in the inner membrane. Finally, the pV determines the switch from RF to the so-called (+)-strand ssDNA synthesis. The pV- complexed DNA is guided to the morphogenic trans-membrane proteins pI, pXI and pIV, where the assembly of the phage particle takes place. These morphogenic proteins share similarities with other bacterial proteins involved in protein export, suggesting a related mechanism for the assembly of filamentous phage and type IV pilus biogenesis (Russel *et al*., 1997). All five structural proteins of the virus particle are anchored in the inner membrane prior to their incorporation into phage particles (Ohkawa and Webster, 1981; Endemann and Model, 1995). DNA bound pV is continuously displaced with pVIII, dependent on thioredoxin (Feng et al., 1997). The morphogenic proteins pI and pXI export the ssDNA, probably by an ATP dependent mechanism, extruding the newly formed particle into the extra-cellular environment (Russel *et al*., 1997; Marvin, 1998). Plaques formed by these phage on an *E. coli* lawn appear turbid, because the infected bacteria are only impaired in growth but not lysed. This distinguishes the filamentous phage from most other bacterial viruses which are icosahedral in shape, accumulate in the cell cytoplasm and accomplish their release from the host cell by lysing it.

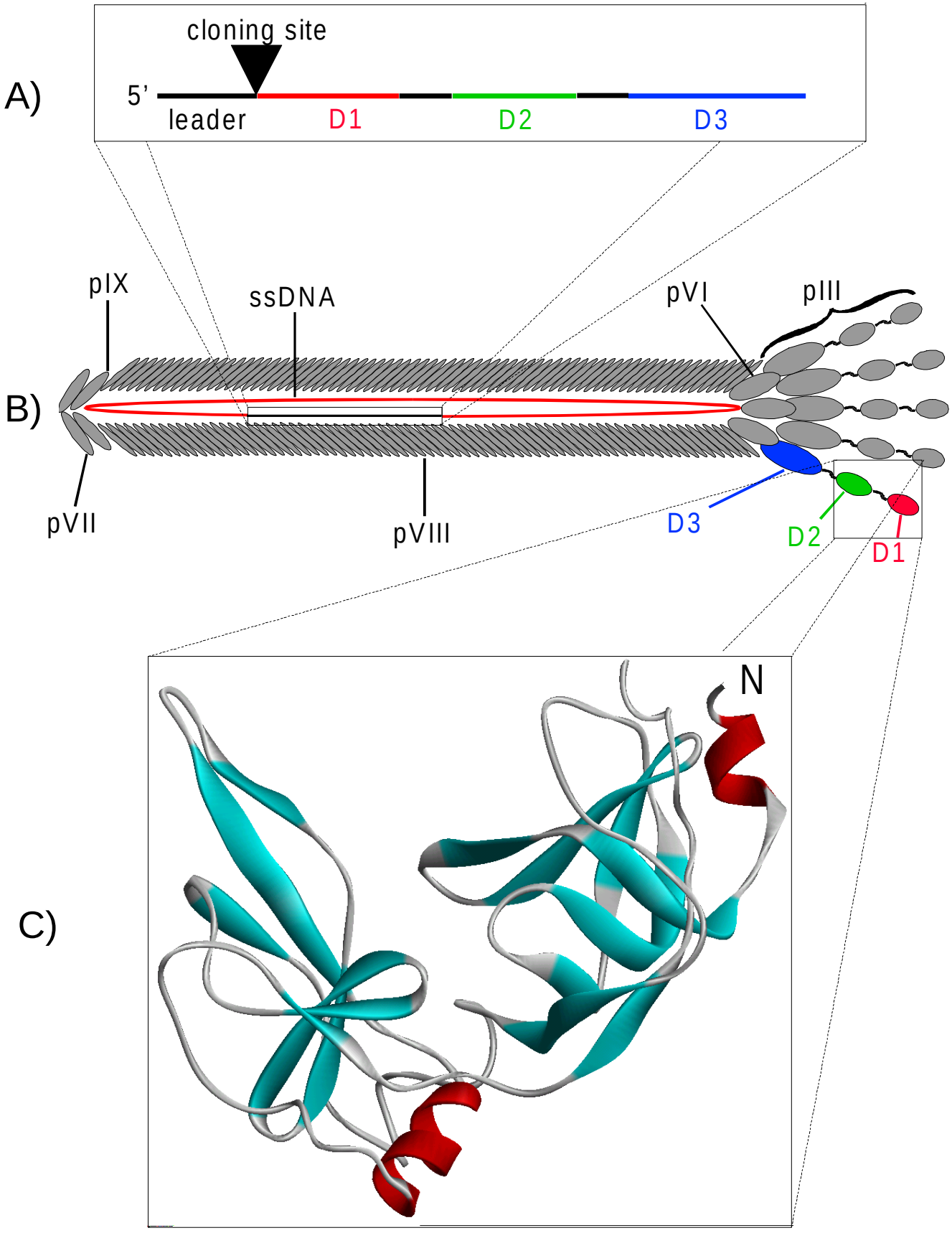


Figure 1.1: Filamentous phage. **A)** pIII gene composition with additional cloning site for introdution of gene-fusions, **B)** schematic drawing with coat proteins and packaged ssDNA, **C)** crystal structure of the two N-terminal domains obtained from 2g3p (Holliger P. and Williams R.L), α-helices are coloured red and β-sheet cyan. Picture generated with WebLab Viewer Lite 3.5

1.1.3 Phage-display systems

For phage-display, peptides or proteins are usually fused to the N-terminus of either minor coat protein pIII or major coat protein pVIII. Additionally, cDNA libraries can be displayed by a fusion to the C-terminus of pVI (Jespers et al., 1995; Fransen et al., 1999). In a rather recent approach, the N-termini of pVII and pIX were used as a fusion partner (Gao *et al*., 1999). Phage particles can either contain a phage genome, or transduce a phagemid which consists of a plasmid carrying the phage origin of replication and one gene encoding a coat protein fusion. A resistance marker gene allows for the selection of library-containing *E. coli* cells for propagation. Phagemids have to be propagated with the aid of a super-infecting helper-phage providing all the necessary genes needed for particle formation but itself being defective in replication. The resulting difference between phage and phagemid for phage-display is the valency of the fusion protein displayed on the surface of the particle. A phage usually possesses 3-5 copies of the pIII and some 3000 copies of the pVIII coat protein, depending on the length of encapsidated genome. With a phagemid, the number of fusion protein copies per phage particle can be adjusted by the promoter preceding the gene. There are several advantages for the use of phagemids, especially if the protein to be displayed is large and/or reduces the infectivity of the phage particles. This could lead to an accumulation of non-displaying deletion phage, elevating the non-specific background in the selection process. Depending on the coat protein used as fusion partner and the choice of the system, different proteins can be effectively displayed. The minor coat protein pIII tolerates N-terminal fusions with random 15mer peptides (Devlin et al. 1990) or proteins as large as scFv (McCafferty *et al*., 1990) and Cytochrome b_{562} (Ku and Schulz, 1995). Many other proteins like protease inhibitors as hPSTI (Röttgen and Collins, 1995) and whole enzymes such as β-lactamase (Soumillion et al., 1994) were displayed on pIII using a phagemid system, alleviating constraints in terms of infectivity, thus leading to a more stable library. This is even more obvious for the major coat protein which tolerates only the insertion of six N-terminal amino acids due to steric hindrance of neighbouring fusion proteins (Greenwood *et al*. 1991). Larger peptides and proteins like Fab, Trypsin, or BPTI were efficiently introduced by the use of hybrid phage producing wild type and fused protein pVIII or using a phagemid system (Greenwood *et al*. 1991; Kang *et al*. 1991; Corey *et al*. 1993; Markland *et al*. 1991). Display of heterologous proteins on filamentous phage coat proteins is limited to secretable variants, which are capable to adopt a native conformation under non-reducing conditions. Therefore, cytoplasmatic proteins containing cysteine residues in their sequence are prone to aggregate in the periplasm and will not be translocated along with the phage particle. There are alternative phage-display systems available employing the λ-phage or T4-phage, which allow the display of cytoplasmatic proteins on the surface (Mikawa *et al*., 1996; Ren *et al*., 1996).

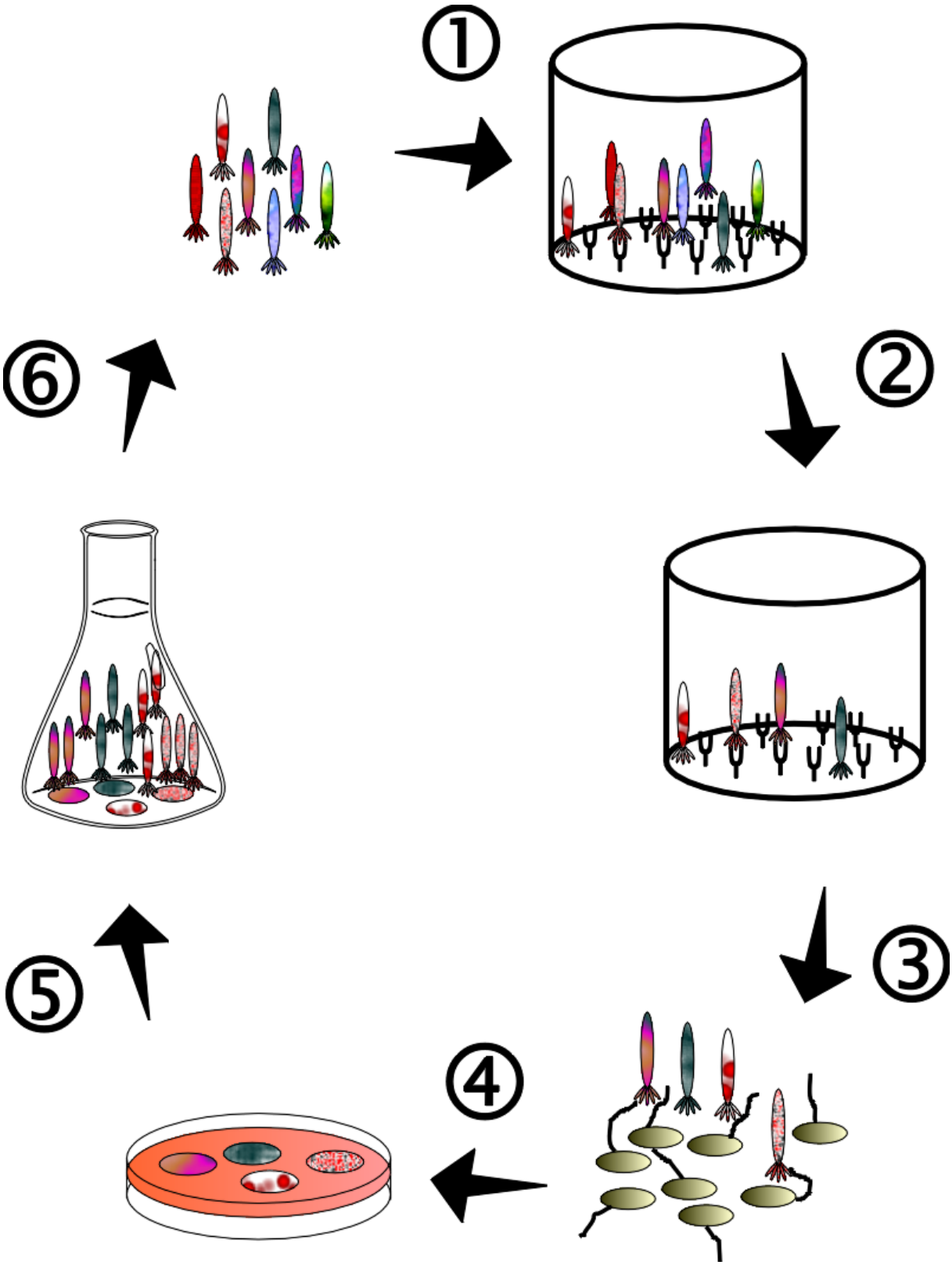


Figure 1.2: Biopanning with filamentous bacteriophage. 1) incubation of phage library with immobilised target 2) panning of binding phage 3) elution of phage and infection of male E. coli 4) selection of infected cells 5) amplification of phage in liquid culture 6) preparation of enriched population

1.1.4 Biopanning

The complete panning cycle is displayed in Figure 1.3 above. The target molecule is often immobilised on a solid support. This can either be a non-specific immobilisation on plastic surfaces like maxisorp microtitre wells and immunotubes, or a specific immobilisation via an antibody or other compound binding to a tag sequence. For the latter case it is advisable to incubate the phage population with the target in solution which enhances the diffusion and perform the capture to a surface later. Unspecific immobilisation can mask the antigen of interest or alter the structure of the immobilised target and lead to false positives in the course of selection. Previous blocking of the solid support with skimmed milk or 3% BSA in buffer solution reduces the background binding of phage which do not recognise the target. It is also advisable to use some blocking agent in solution during the initial incubation of the phage with the target. After a longer incubation period, several washing steps are performed to select for the correct binding variants. Elution of these variants is often performed unspecifically by the addition of an acidic buffer or direct infection of *E. coli in situ*. If available, competitive ligands can be used as an alternative, promoting the elution of specific phage. For the propagation and enrichment of the target binding variants, the eluted phage are allowed to infect *E. coli* cells which are grown under antibiotic selection either separately on a petri dish, or subjected directly to an erlenmeyer flask. The selection on a petri dish enables clones to separately form colonies which are otherwise superseded by competing clones in a liquid culture. After colony formation on the petri dish, the cells are resuspended and pooled in an erlenmeyer flask. In the case of a phagemid system, *E. coli* has to be super-infected by the helper phage to initiate the phage particle production. The produced phage are then harvested by PEG/salt precipitation and resuspended in the incubation buffer to start the next cycle of panning. As the titres of the input and elution populations should be determined, the enrichment of phage can easily monitored by comparison with a parallel control panning. Usually, three to five cycles of panning and propagation are necessary to enrich for well binding clones which can then be isolated and sequenced. Stringency can be increased on binding by various methods over the selection rounds if avidly binding variants are desired. The alignment of similar sequences obtained allows the design of a consensus motif which may represent the best binding variant for the given target.

1.2 Affinity Purification

Protein purification is a necessary technique to make proteins available to functional studies or medical applications, for which raw extracts cannot be used. Classical protein purification involves a multiplicity of different separation steps, usually resulting in low yields of pure proteins consuming time and material. Monoclonal antibodies allow a

high selectivity with affinity chromatography but are costly and bound molecules are difficult to release. A method which is cheap, simple and selective at the same time is Immobilised Metal Affinity Chromatography, or IMAC. With this technique, a specific interaction of certain peptides with immobilised metal ions is exploited to obtain highly homogenous proteins or protein fusions in a single purification step. Such a purification is applicable to both analytical and large-scale separations. Metal complexes are stable under a variety of conditions and can be recycled many times. Elution of bound proteins can be achieved under mild conditions, thus keeping the protein in a native state. Even denatured proteins can successfully be bound and refolded on IMAC columns (Zahn *et al.*, 1997). Originally, IMAC was developed to separate heavy metal binding proteins from blood serum (Porath *et al.*, 1975). The basic principle of IMAC involves rapidly reversible interactions with metal ions immobilised on a chromatographic support (e.g. Cu^{2+} bound by iminodiacetate, IDA) resulting in the retention of proteins with metal-coordinating ligands on their surface. Mainly histidines with their imidazole side-chain form the interaction with the metal at a neutral pH. Elution can be achieved using different protocols, depending on the microenvironment of the histidines, determining the strength of the histidine-metal interaction. Either a gradient or stepwise lowering of the pH to 4 or the addition of imidazole up to .5M at neutral pH releases bound proteins from the metal-complexes. This allows a highly group-specific separation of proteins even from crude extracts.

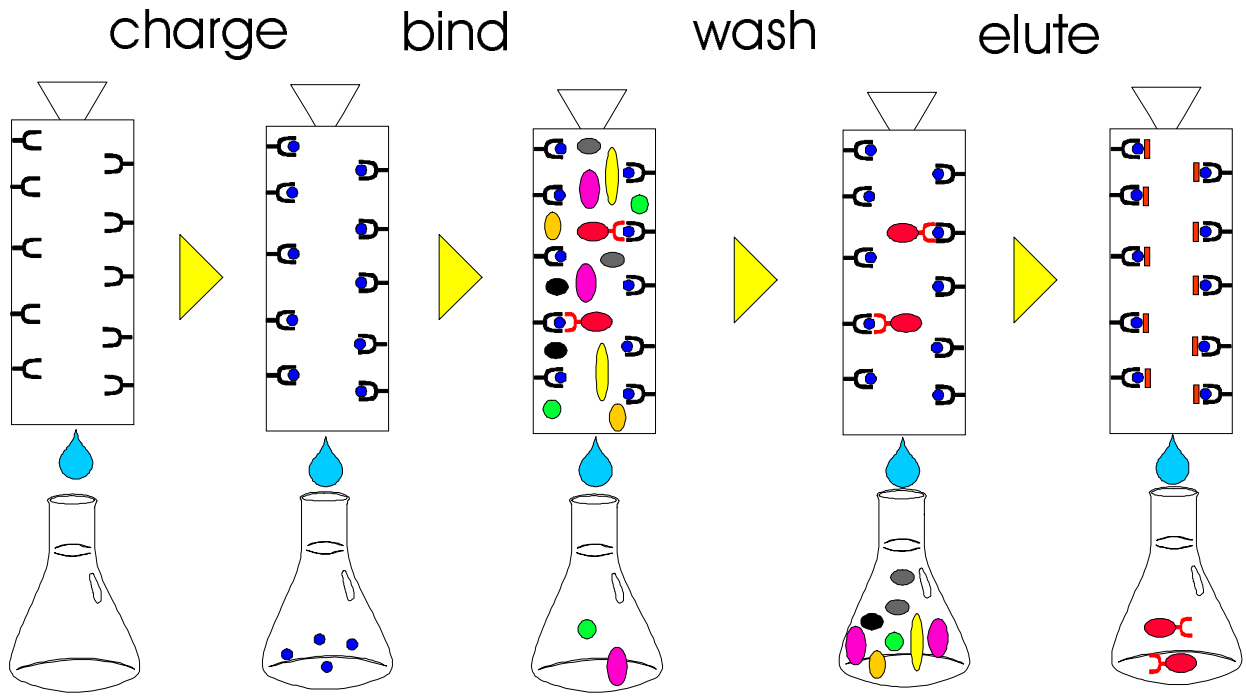


Figure 1.3: IMAC purification scheme. Metal ions are symbolized as blue spheres, chelators as horseshoe magnets, the recombinant fusion protein is coloured red.

Most of the naturally occurring proteins have only moderate affinities for metal-complexes, especially under high ionic strength conditions suppressing possible electrostatic interactions. Therefore, a recombinant protein can easily be engineered by the fusion with histidine-rich affinity-"handles" (Hochuli *et al.*, 1988). Using different metal-ions, chelating agents, and solvent conditions, a procedure can be tailored to specifically purify such a recombinant protein. The strength of protein adsorption for the immobilised transition metal-ions increases with the following order $Co^{2+}<Zn^{2+}<Ni^{2+}<Cu^{2+}$ on IDA materials (Winzerling *et al.*, 1992). The use of different chelating supports determines the stability of the metal complex under different conditions and the affinity of the proteins to be purified (Jiang *et al.*, 1998). A tetradentate chelator such as nitrilotriacetate (NTA) is more resistant towards a chaotropic salt and leeches less metal ions as a tridentate chelator like IDA (see Figure 1.4A and B).

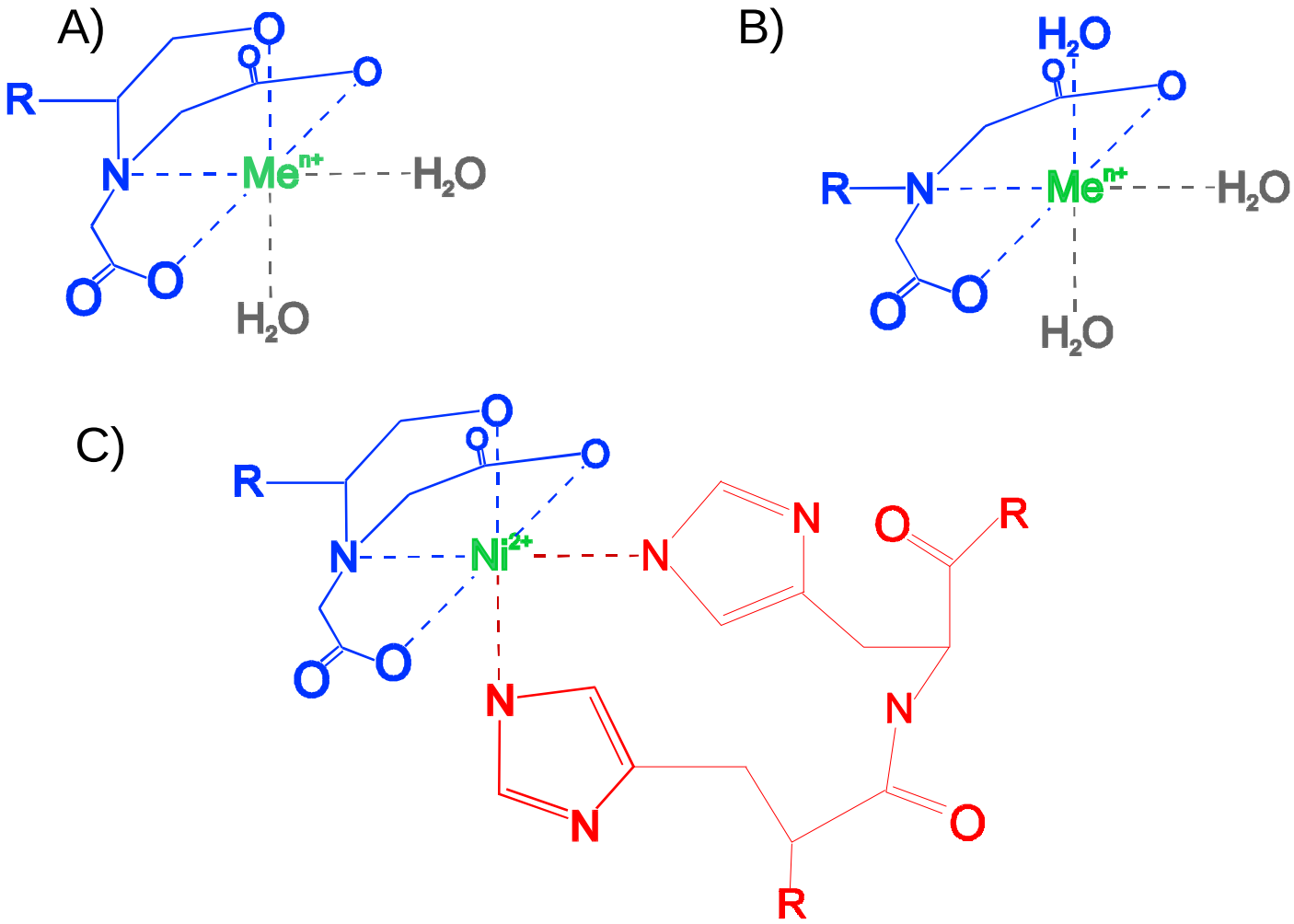


Figure 1.4: Metal ion complexes and interaction with His-tags. **A)** tetradentate nitrilotriacetic acid (NTA), **B)** tridentate iminodiacetic acid (IDA), **C)** Ni-NTA complex with two histidine residues of a His-tag

Buffers containing Tricine, citrate or Tris should be avoided, since they also have metal-chelating properties and could remove the metal-ions from the solid support. Many recombinant expression vectors contain a hexahistidine coding sequence close to the multiple cloning site, readily engineered for IMAC purification of the expressed recombinant protein. The use of such expression/purification systems has led to a more rapid detection and analysis of interesting proteins (Kelman *et al.*, 1995). Combination of several features on one tag sequence allow the purification of difficult peptides and proteins (Dobeli, 1998). Detection of histidine-tags can now be achieved by specific monoclonal antibodies, a biotin-NTA or peroxidase-NTA conjugate (O'Shawnessy et al., 1995; Jin et al., 1995;). The standardisation of recombinant proteins *via* histidine-tags can finally be exploited for high-throughput techniques like antibody screening of protein microarrays (Lueking *et al.*, 1999).

Hard Lewis metal ions such as Fe^{3+}, Al^{3+}, Ca^{2+} and Mg^{2+} have also been applied to IMAC. The first two were shown to bind primary phosphate groups as found on phosphoproteins and nucleotides (Andersson and Porath, 1986; Andersson, 1991). Especially Fe^{3+} seems to be highly selective under mild acidic conditions (pH4-6) and thus often used to separate phosphorylated isoforms of enzymes and peptides (Neville *et al.*, 1997). The specificity of interaction is also exploited for detection with a peroxidase-chelate-Fe^{3+} conjugate. The other metal-ions Ca^{2+} and Mg^{2+} mainly bind to carboxyl groups (Zachariou and Hearn, 1996).

1.3 Aim of this Work

Although phage-display finds increasingly more applications, only a few attempts have been made to find affinity handles for protein purification. As IMAC offers the advantage of using cheap materials, simple procedures, and selective binding of short peptide-sequences, it is a good target for panning a phage peptide library. Finding a new affinity handle as good as the well known His-tag should be of commercial interest, since patents can be circumvented. Several attempts were made using conventional oriented peptide libraries on cellulose (Kramer *et al.* 1993) and site-directed mutagenesis of proteins (Arnold and Haymore, 1991). Finding affinity-tags for other metal ions than Cu^{2+}, Ni^{2+}, Zn^{2+} and Co^{2+} would probably produce novel sequences providing the specificity. Peptide ligands recognising non-toxic metals would be advantageous for the purification of pharmaceutical products and reduce the payload on the environment.

At the beginning of this work, only one publication on phage display in combination with IMAC was available (Barbas *et al.*, 1993). Several scFv variants were identified, binding to metal-chelates in a specific manner. However, affinity handles of the size of

short peptides are less likely than complete protein domains to impair the expression and folding of a fusion protein. Therefore, the most suitable phage peptide library available for the experiments was M13LP67, based on M13 phage with an additional ampicillin resistance and a 15-mer random amino acid insertion at the N-terminus of the minor coat protein pIII (Devlin *et al*. 1990).

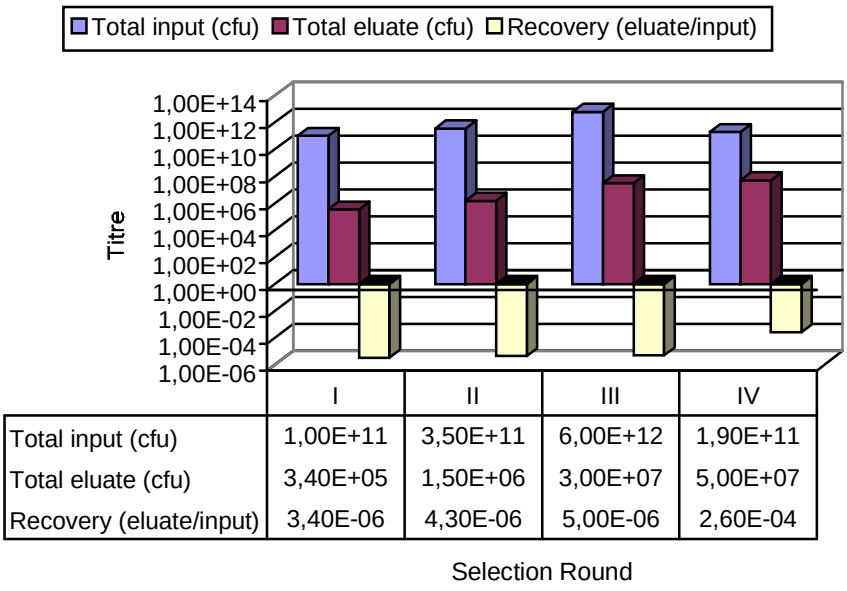
2 RESULTS

2.1 Selection using IDA-immobilised metals

2.1.1 Affinity selection of transition metal ion binding peptide variants

As mentioned in the introduction, conditions compatible with the conventional IMAC were chosen in order to facilitate the panning. This applies to the PBS buffer as it contains .5M of sodium chloride to suppress ionic interactions at a neutral pH. The affinity material of choice were the SpinZyme affinity separation units provided by Pierce. The separation unit consists of a bucket with a porous IDA-cellulose membrane at the bottom inserted in an eppendorf tube. The advantage compared to other affinity materials such as chelating sepharose is the minimal void volume which should decrease the background and the simplicity in terms of handling, since separation can be achieved by centrifugation. As the IDA-membrane comes already complexed with iron(III), the metal has to be removed before charging it with the transition metal. In order to visualise the success of a panning, titres of total input and eluted phage particles were compared and displayed in diagrams.

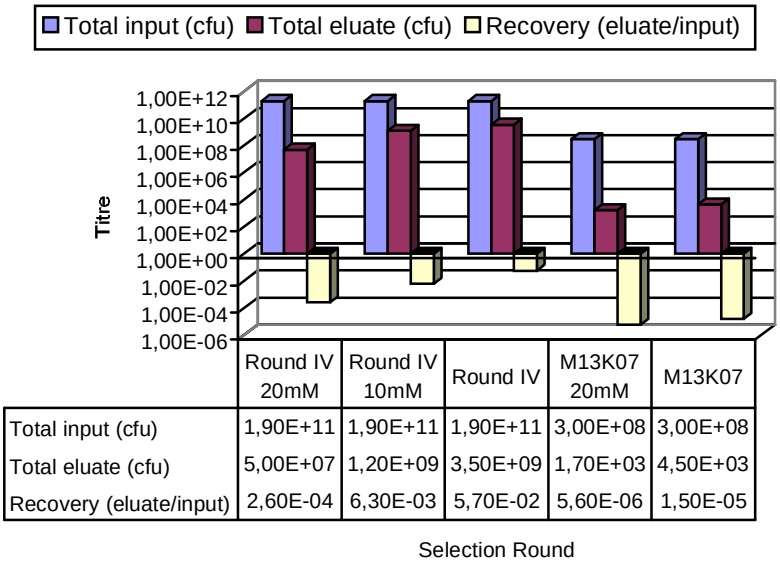
2.1.1.1 Cobalt(II) selection



	I	II	III	IV
Total input (cfu)	1,00E+11	3,50E+11	6,00E+12	1,90E+11
Total eluate (cfu)	3,40E+05	1,50E+06	3,00E+07	5,00E+07
Recovery (eluate/input)	3,40E-06	4,30E-06	5,00E-06	2,60E-04

Figure 2.1: Co(II) SpinZyme selection

During the affinity selection of the first three rounds, the recovery of phage in terms of eluate divided by input titres, stays more or less constant. Only in the fourth round an enrichment compared to the previous ones becomes obvious. To ensure that this is due to specific binding of the phage population amplified from the eluate of the third round, control pannings were carried through. As the stringency of washing was increased during the rounds by additional washing steps and the addition of the competitive ligand imidazole, varying concentrations of this ligand were applied. The non-displaying helper phage M13K07 serves as an additional control.



	Round IV 20mM	Round IV 10mM	Round IV	M13K07 20mM	M13K07
Total input (cfu)	1,90E+11	1,90E+11	1,90E+11	3,00E+08	3,00E+08
Total eluate (cfu)	5,00E+07	1,20E+09	3,50E+09	1,70E+03	4,50E+03
Recovery (eluate/input)	2,60E-04	6,30E-03	5,70E-02	5,60E-06	1,50E-05

Figure 2.2: Co(II) SpinZyme control

The stringency imposed by imidazole becomes clearly visible if one compares the round IV population eluate with 20mM imidazole from the panning and the controls with 10mM imidazole and without. A factor above 100 can be observed. The M13K07 helper phage is also affected by the imidazole concentration in the washing buffer, but only by more than two fold. The difference can be attributed to a competitive binding of several amino acids present in the round IV phage pool. Especially if one compares the yield of recovery between the round IV population and the helper phage at 20mM and without imidazole, differing by a factor of about 50 and 4000 respectively. Therefore several individual clones obtained from the panning after the fourth cycle were picked and subjected to DNA sequencing of the fusion protein.

As already expected from known transition metal binding peptides, histidine is the most prominent amino acid in these sequences. Though the stringency was high in the last two rounds of panning, 2 out of 20 sequenced clones are deletions, having lost the insert

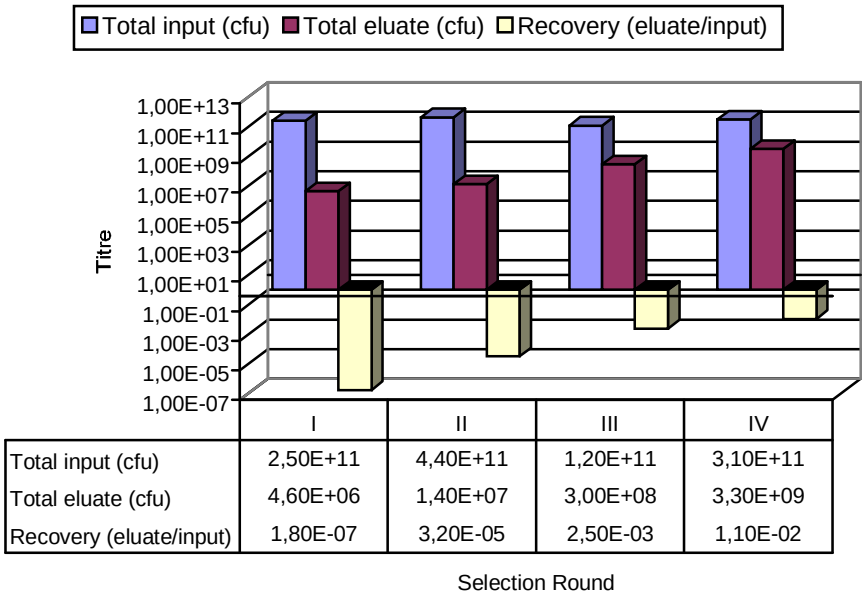
including the proline rich linker between the leader sequence and the mature pIII minor coat protein.

Table 2.1: Sequences of obtained from Co(II) SpinZyme selection

Clone number	Insert sequence	Frequency
CoSZIV#1	T **H** S T **H** P A S **H** **H** R **H** K **H** T	9
CoSZIV#7	**H** R **H** **H** R P **H** E **H** S **H** R V T P	3
CoSZIV#4	A L P R S S P **H** **H** **H** **H** L P **H** R	3
CoSZIV#5	M G S N **H** M **H** **H** **H** **H** F P **H** L P	2
CoSZIV#11	P **H** Q G Y **H** K A T **H** **H** **H** W S P	1
CoSZIV#2	deletion	2

2.1.1.2 Nickel(II) selection

Since the panning on cobalt(II) was successful, the same conditions were chosen for the panning on nickel(II). This includes the charging of the material, the buffers and washing procedures.



	I	II	III	IV
Total input (cfu)	2,50E+11	4,40E+11	1,20E+11	3,10E+11
Total eluate (cfu)	4,60E+06	1,40E+07	3,00E+08	3,30E+09
Recovery (eluate/input)	1,80E-07	3,20E-05	2,50E-03	1,10E-02

Figure 2.3: Ni(II) SpinZyme selection

Controls were made along with the cross-reactivity tests described in another chapter. Of the 4th cycle, 5 individual clones were picked for sequencing.

Table 2.2: Sequences obtained from Ni(II) SpinZyme selection

Clone number	Insert sequence	Frequency
NiSZIV#20	A Y P **H** F **H** S N S **H** L I **H** S **H**	2
NiSZIV#18	Y **H** T S I **H** **H** **H** **H** P V D **H** L A	1
NiSZIV#16	L D **H** T Y R A **H** S K V **H** **H** **H** **H**	1
NiSZIV#17	A P S **H** **H** T **H** S **H** **H** L T Q M A	1

Of the 5 clones sequenced, no deletion was observed.

2.1.1.3 Zinc(II) selection

Again, the same condition as above were chosen for the affinity selection with zinc(II).

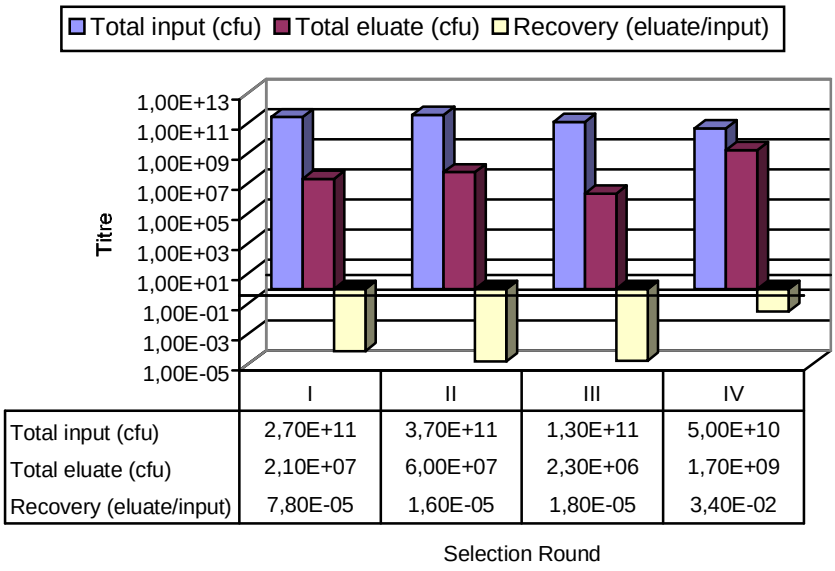


Figure 3.4: Zn(II) SpinZyme selection

Five individual clones from the 4th selection round were picked and subjected to DNA-sequencing of the insert.

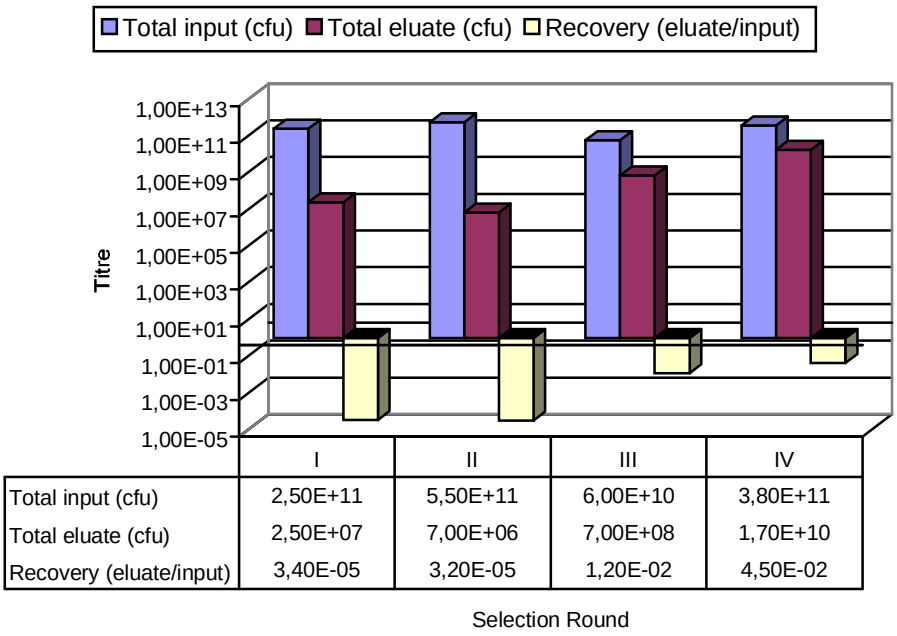
Table 2.3: Sequences obtained from Zn(II) SpinZyme selection

Clone number	Insert sequence	Frequency
ZnSZIV#1	**H** R **H** **H** R P **H** E **H** S **H** R V T P	4
ZnSZIV#2	M G S N **H** M **H** **H** **H** **H** F P **H** L P	1

Both sequences occurred in the affinity selection with cobalt(II). There seems to be a limited number of clones in the initial library pool which are able to bind to transition metal chelates and have only a limited selectivity. In the 4th round, a clear enrichment can be observed for the clone ZnSZIV#1.

2.1.1.4 Copper(II) selection

Comparable to the the selection on zinc(II) and cobalt(II), identical clones show up with the selection on copper(II). Clone CuSZIV#12 is present in both of the other affinity selections, whereas clone CuSZIV#13 is found only in the cobalt(II) selection. Surprisingly, no enrichment of the selected clones were observed with copper(II) even after 4 cycles of panning.



	I	II	III	IV
Total input (cfu)	2,50E+11	5,50E+11	6,00E+10	3,80E+11
Total eluate (cfu)	2,50E+07	7,00E+06	7,00E+08	1,70E+10
Recovery (eluate/input)	3,40E-05	3,20E-05	1,20E-02	4,50E-02

Figure 2.5: Cu(II) SpinZyme selection

Table 2.4: Sequences obtained from Cu(II) SpinZyme selection

Clone number	Insert sequence	Frequency
CuSZIV#13	M G S N **H** M **H** **H** **H** **H** F P **H** L P	1
CuSZIV#12	**H** R **H** **H** R P **H** E **H** S **H** R V T P	1
CuSZIV#15	K **H** **H** L **H** **H** E **H** A Y P T L K N	1
CuSZIV#14	**H** R S W T S P **H** N **H** P **H** T **H** **H**	1
CuSZIV#11	A **H** P **H** R **H** **H** S D S M L V T **H**	1

2.1.2 Affinity selection of hard Lewis acid binding peptide variants

2.1.2.1 Aluminium(III) selection

The first panning trial with aluminium(III) as a ligand, the same conditions as for the transition metals were applied. At the second and 3rd round, a washing step with incubation buffer containing 3%BSA was added. One important difference was the elution buffer used. Since the mode of binding of peptides to the metal ion may differ from the transition metals, imidazole cannot be assumed to be the appropriate eluent for the phage variants. Therefore, EDTA was used to remove the metal ions from the chelating support. A concentration of .05M EDTA was found not to interfere severely with the elution and re-infection process.

Clones picked for sequencing did not contain an insert sequence. Therefore, the panning conditions did not allow the screening for specifically binding variants. As aluminium(III) is also known to bind phosphoproteins, it may be that the phosphate containing PBS buffer is not compatible with the affinity of the ligand with the metal ion. Also the relatively high salt content (.5M NaCl) could inhibit binding mediated by pseudocation exchange adsorption. Due to these assumptions, a different approach was made. The buffer of choice was now MOPS, reported to be non-chelating.

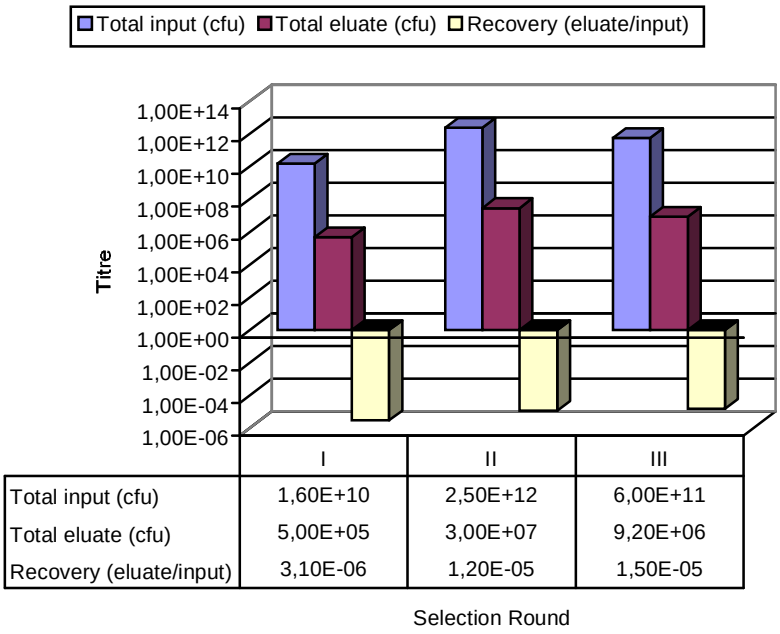
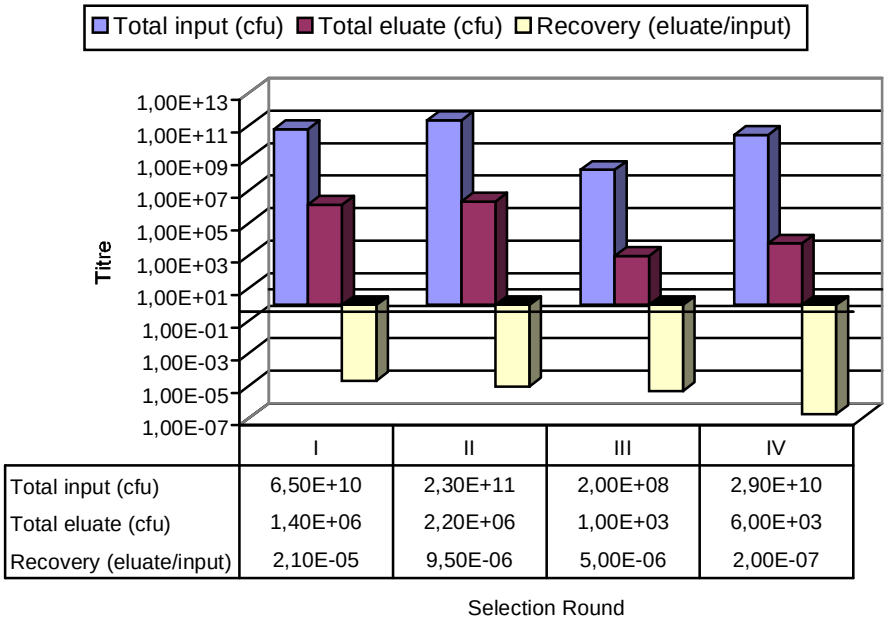


Figure 2.6: Al(III) SpinZyme selection (PBS)

This time, four rounds were performed to select for specifically binding variants.



	I	II	III	IV
Total input (cfu)	6,50E+10	2,30E+11	2,00E+08	2,90E+10
Total eluate (cfu)	1,40E+06	2,20E+06	1,00E+03	6,00E+03
Recovery (eluate/input)	2,10E-05	9,50E-06	5,00E-06	2,00E-07

Figure 2.7: Al(III) SpinZyme selection (MOPS)

Though the recovery of phage looked discouraging in the second approach, 5 clones were picked and assayed by restriction analysis for an insert. One clone was found to carry an insert and was sequenced.

Table 2.5: Sequences obtained from Al(III) SpinZyme selection

Clone number	Insert sequence	Frequency
AlSZIV#4	Q A L F S S N F S F R G R L A	1
	deletions	4

In order to verify the specific binding of this clone, a control panning was performed on Al(III) complexed SpinZyme. Washing and incubation conditions were identical to the previous screening procedures of the 1st selection round.

No enrichment can be observed for the single clone AlSZIV#4 above the initial panning round I. Hence, the sequence found in this clone cannot be attributed to a specific binding to the affinity material.

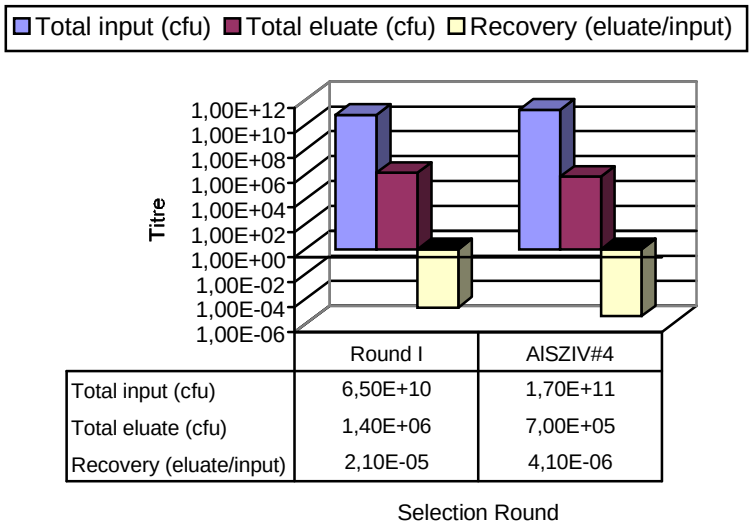
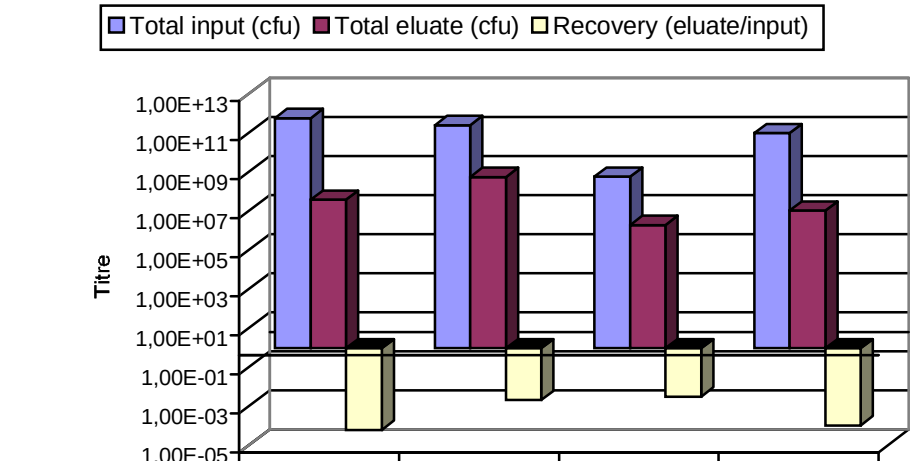


Figure 2.8: Al(III) SpinZyme control

2.1.2.2 Iron(III) selection

Since the SpinZyme affinity separation units come readily complexed with iron(III) it is the easiest and best controlled material for the selection process.



	I	II	III	IV
Total input (cfu)	6,00E+11	2,60E+11	6,00E+08	1,00E+11
Total eluate (cfu)	3,90E+07	5,80E+08	2,00E+06	1,10E+07
Recovery (eluate/input)	6,50E-05	2,20E-03	3,30E-03	1,10E-04

Selection Round

Figure 2.9: Fe(III) SpinZyme selection

In contrast to the panning on Al(III), a clear enrichment can be observed starting with already the 2nd cycle. Individual clones were picked and assayed for deletions by restriction analysis. Six of 15 clones were found to have lost their insert. The remaining nine clones were sequenced.

Table 2.6: Sequences obtained from Fe(III) SpinZyme selection

Clone number	Insert sequence	Frequency
FeSZIV#4	G I P A **H** E Q **H** T **K** **K** L W L L	4
FeSZIV#1	W P T **K** **K** F T L T **H** **K** **H** S **K** R	2
FeSZIV#7	A **H** P S **H** **H** R A P S R **H** **K** S I	2
FeSZIV#14	L Q S F G **K** L P Y S R L Y S V	1
	deletions	9

Control pannings were conducted to verify the specificity of the clones

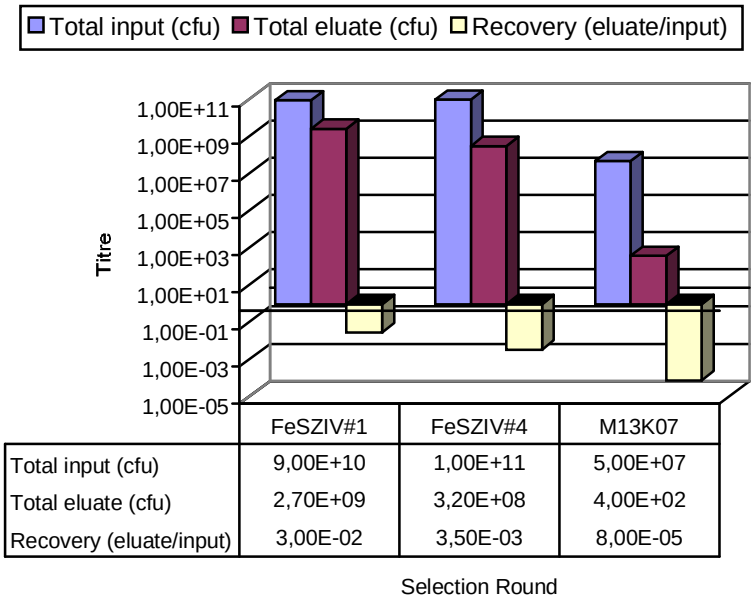
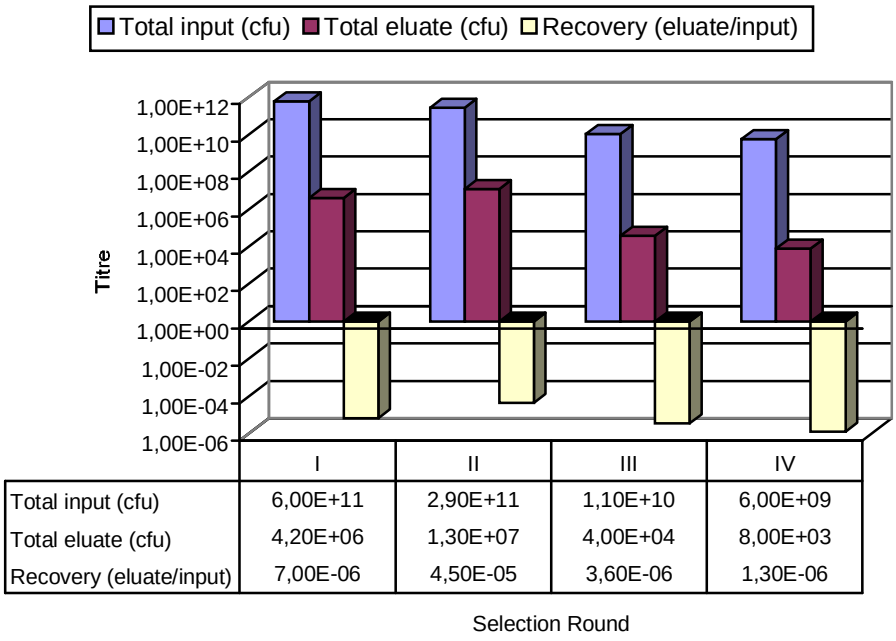


Figure 2.10: Fe (III) SpinZyme control

These controls indicate a selective and specific binding of the clones FeSZIV#1 and #4 in contrast to the helper phage M13K07 without the displayed sequences. This is very much comparable to the results obtained from the panning on the transition metal ions.

2.1.2.3 Magnesium(II) selection

As many naturally occurring proteins bind magnesium, finding a specific sequence involved without the requirement of sterical constraints could offer interesting perspectives, also for protein purification. The panning conditions and buffers were identical to those conducted with iron(III).



	I	II	III	IV
Total input (cfu)	6,00E+11	2,90E+11	1,10E+10	6,00E+09
Total eluate (cfu)	4,20E+06	1,30E+07	4,00E+04	8,00E+03
Recovery (eluate/input)	7,00E-06	4,50E-05	3,60E-06	1,30E-06

Figure 2.11: Mg(II) SpinZyme selection

Of the five single clones picked, only one was shown to contain an insert by restriction analysis.

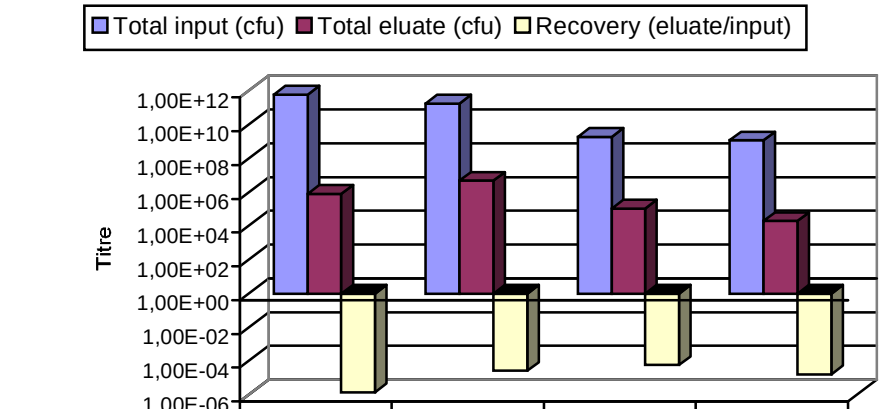
Table 2.7: Sequences obtained from Mg(II) SpinZyme selection

Clone number	Insert sequence	Frequency
MgSZIV#3	G T S K A F W S G Q P L T Y S	1
	deletions	4

A similar result as from the Al(III) panning without specificity of the single insert containing clone evaluated.

2.1.2.4 Calcium(II) selection

Conditions in panning and buffers were kept identical to those performed with Mg(II) and Fe(III).



	I	II	III	IV
Total input (cfu)	6,00E+11	1,80E+11	1,90E+09	1,20E+09
Total eluate (cfu)	8,20E+05	4,90E+06	1,10E+05	2,00E+04
Recovery (eluate/input)	1,40E-06	2,70E-05	5,80E-05	1,70E-05

Selection Round

Figure 2.12: Ca(II) SpinZyme selection

Single clones from the 4th selection round were examined by restriction analysis. All five were shown to contain only deletions. This indicates that calcium is not an adequate ligand for short peptide sequences.

2.1.2.5 Cerium(IV) selection

The panning conditions were identical to those conducted with Al(III) and PBS.

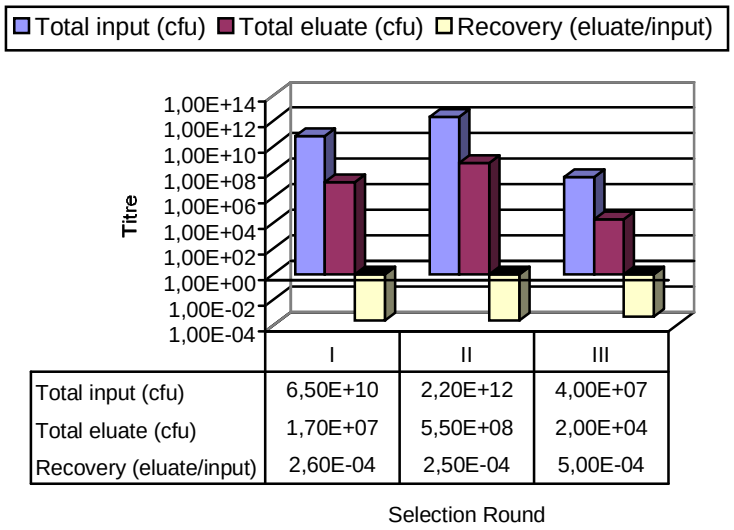
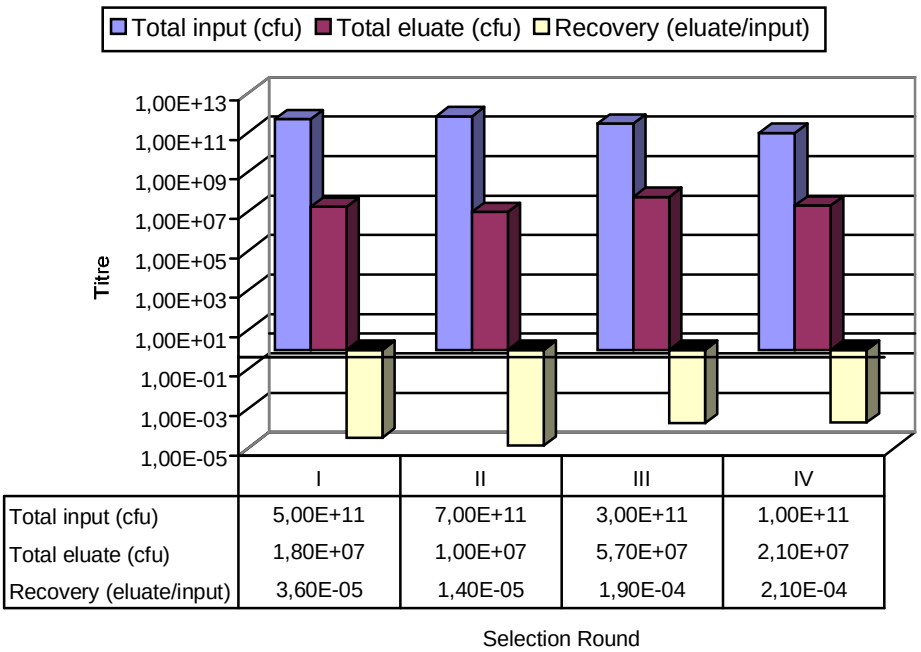


Figure 2.13: Ce(IV) SpinZyme selection

The panning was aborted at this point, because the background binding was by far too high to achieve a good selection of specific binders.

2.1.3 Titanium(IV) selection

Titanium does not belong to the hard Lewis acids and its highly charged ion Ti^{4+} is usually unstable under aqueous and oxidising conditions. Therefore, the ion was applied to the chelating matrix in the organic solvent it was delivered in. The idea was that a sufficient amount of ion is complexed to IDA, stabilising the ion in aqueous buffer solutions such as MOPS. Panning conditions and buffers were identical to those applied for the transition metal selections. Elution was achieved by addition of a glycine buffer at pH2.2 for 20 minutes and subsequent neutralisation before re-infection of *E. coli*.



	I	II	III	IV
Total input (cfu)	5,00E+11	7,00E+11	3,00E+11	1,00E+11
Total eluate (cfu)	1,80E+07	1,00E+07	5,70E+07	2,10E+07
Recovery (eluate/input)	3,60E-05	1,40E-05	1,90E-04	2,10E-04

Figure 2.14: Ti(IV) SpinZyme selection

No real enrichment can be deduced from these selections. Restriction analysis of five individually picked clones revealed only one clone containing an insert.

Table 2.8: Sequences obtained from Ti(IV) SpinZyme selection

Clone number	Insert sequence	Frequency
TiSZIV#2	M P S S L P N Y S W H M L S V	1
	deletions	4

Because this result seemed identical to those obtained previously with panning on Al(III) and Mg(II), no further investigations involving the specificity of this clone were made.

2.1.4 Uncharged SpinZyme control panning

Residual iron(III) on the matrix of SpinZyme or other features of the membrane could act as a bias and enrich clones which are not selective for the metal ion intended. To evaluate this possibility, a selection was performed on the iron(III) stripped SpinZyme affinity separation units. The panning procedures were identical to those in the hard Lewis acid selections.

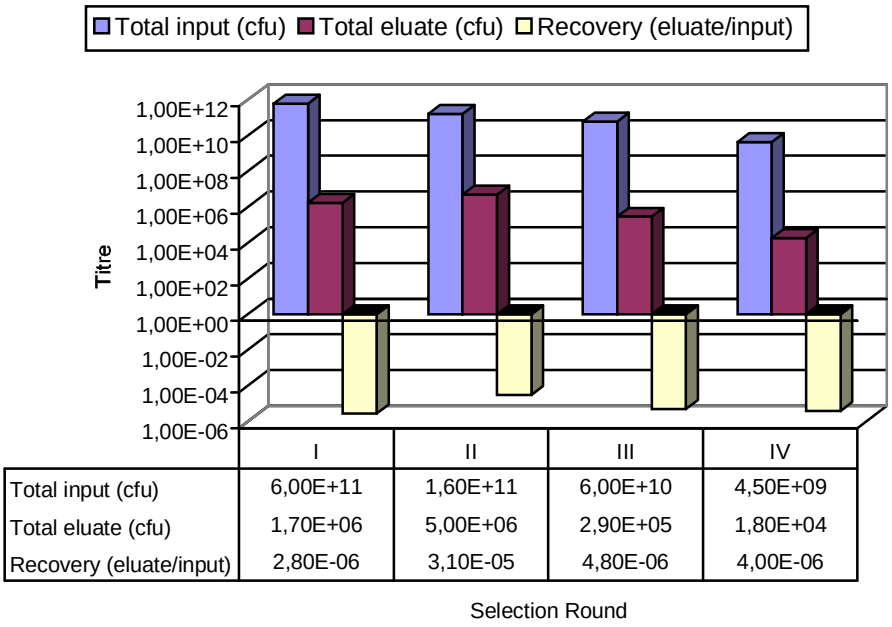


Figure 2.15: SpinZyme negative control

All five clones picked from the 4th round were found to represent deletions.

2.2 Selection using INDIA™-immobilised metals

The purchased affinity material comes in the shape of a microtitre plate, offering a simple handling of manifold samples at a time. Many protocols already exist for the panning of phage libraries on microtitre plates. Because of the previous experiments using the SpinZyme separation units were successful, basically all the buffers were identical. Due to the even smaller void volume of microtitre plates compared to SpinZyme, less washing steps were performed during the selection processes.

2.2.1 Affinity selection of transition metal ion binding peptide variants

All panning conditions are kept the same as for the selections on transition metals bound to SpinZyme.

2.2.1.1 Cobalt(II) selection

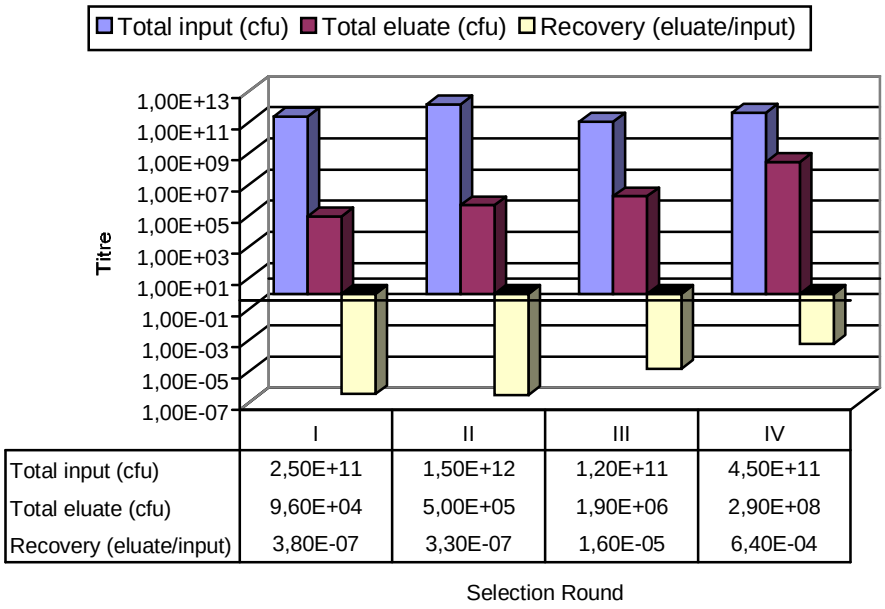


Figure 2.16: Co(II) ReactiBind selection

An enrichment of more than 3 degrees in magnitude can be observed from the 1st to the 4th round of selection. Five clones were picked and subjected to DNA sequencing.

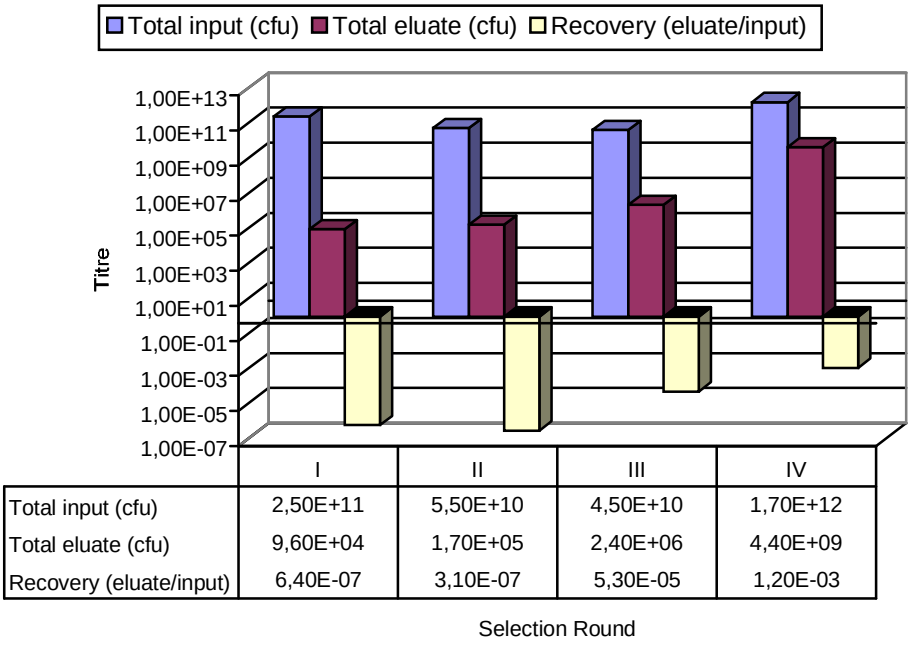
Table 2.9: Sequences obtained from Co(II) ReactiBind selection

Clone number	Insert sequence	Frequency
CoRBIV#1	A **H** Q Q T **H** **H** Y F T **H** **H** L N W	3
CoRBIV#3	V A **H** **H** W W **H** D G Y K **H** P L N	1
CoRBIV#4	**H** R **H** **H** R P **H** E **H** S **H** R V T P	1

The last clone has appeared in the Co(II) selection on SpinZyme before.

2.2.1.2 Nickel(II) selection

It should be noted that for the panning on nickel(II) INDIA no exchange of the metal ion from the support was necessary as ReactiBind comes readily complexed with nickel(II) from the supplier.



	I	II	III	IV
Total input (cfu)	2,50E+11	5,50E+10	4,50E+10	1,70E+12
Total eluate (cfu)	9,60E+04	1,70E+05	2,40E+06	4,40E+09
Recovery (eluate/input)	6,40E-07	3,10E-07	5,30E-05	1,20E-03

Figure 2.17: Ni(II) ReactiBind selection

Compared with the initial round, an enrichment factor of about 5000 can be seen in the 4th cycle of panning. Again, 5 clones were picked and sequenced.

Table 2.10: Sequences obtained from Ni(II) ReactiBind selection

Clone number	Insert sequence	Frequency
NiRBIV#1	**H H H H** S Y M S S I P S T A W	5

All sequenced clones share the same sequence.

2.2.1.3 Zinc(II) selection

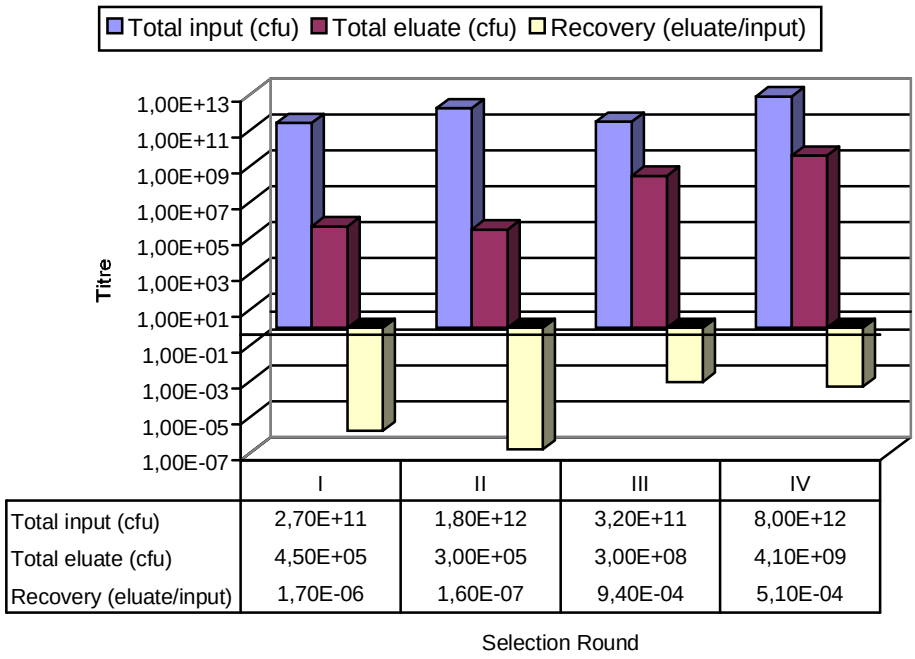


Figure 2.18: Zn(II) ReactiBind selection

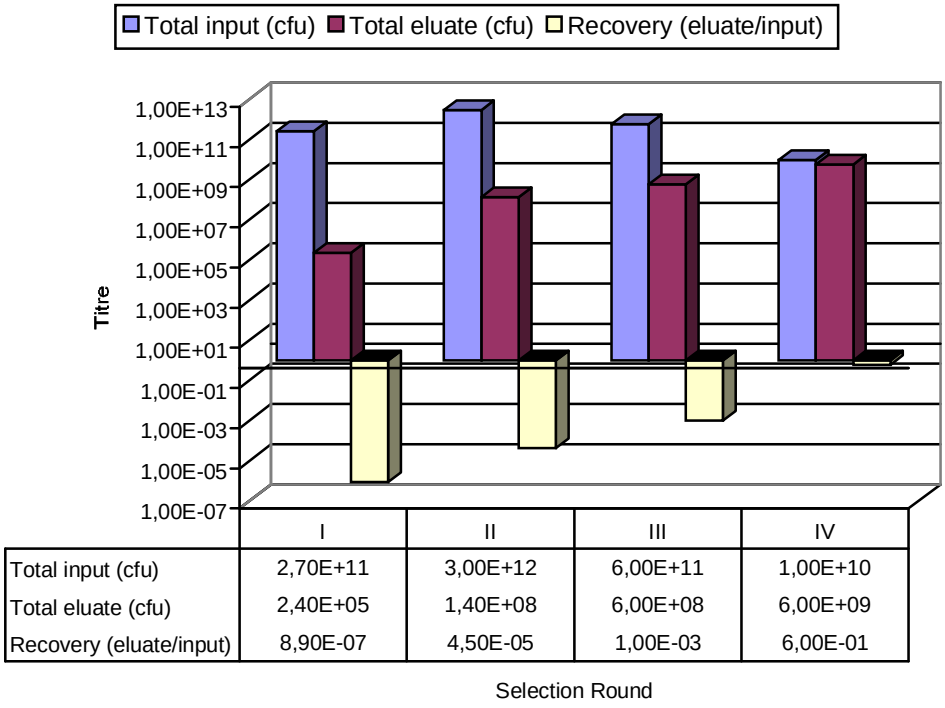
The enrichment over the selection rounds by a factor of about 300 is not as pronounced as with the other transition metal ions. Five individual clones were subjected to DNA sequencing.

Table 2.11: Sequences obtained from Zn(II) ReactiBind selection

Clone number	Insert sequence	Frequency
ZnRBIV#6	**H H H H** S Y M S S I P S T A **W**	3
ZnRBIV#2	**H** R **H H** R P **H** E **H** S **H** R V T P	2

The first sequence turned up as a dominant clone in the selection on Ni(II) ReactiBind, whereas the second sequence was found in several selections on transition metals such as Co(II) SpinZyme.

2.2.1.4 Copper(II) selection



	I	II	III	IV
Total input (cfu)	2,70E+11	3,00E+12	6,00E+11	1,00E+10
Total eluate (cfu)	2,40E+05	1,40E+08	6,00E+08	6,00E+09
Recovery (eluate/input)	8,90E-07	4,50E-05	1,00E-03	6,00E-01

Figure 2.19: Cu(II) ReactiBind selection

This is best enrichment observed so far. About six orders of magnitude are between the recovery ratios of the 1st and 4th round.

Table 2.12: Sequences obtained from Cu(II) ReactiBind selection

Clone number	Insert sequence	Frequency
CuRBIV#1	**H H H H** S Y M S S I P S T A W	2
CuRBIV#2	**H** R **H H** R P **H** E **H** S **H** R V T P	2
CuRBIV#4	A **H** Q Q T **H H** Y F T **H H** L N W	1

Similar to the Zn(II) ReactiBind selection all the sequences did appear in other selection experiments. CuRBIV#1 was selected with both Zn(II) and Ni(II) on ReactiBind, CuRBIV#2 appeared in many other selections as on Co(II) on SpinZyme and finally CuRBIV#4 was selected from Co(II) on ReactiBind.

2.2.2 Other metal ions

2.2.3 Titanium(IV) selection

As described for SpinZyme previously, the panning procedures for Ti(IV) on ReactiBind were almost identical to those applied for the transition metals. The important difference was an additional washing step with 400µl H20 prior to elution achieved by the addition of a glycine buffer pH2.2.

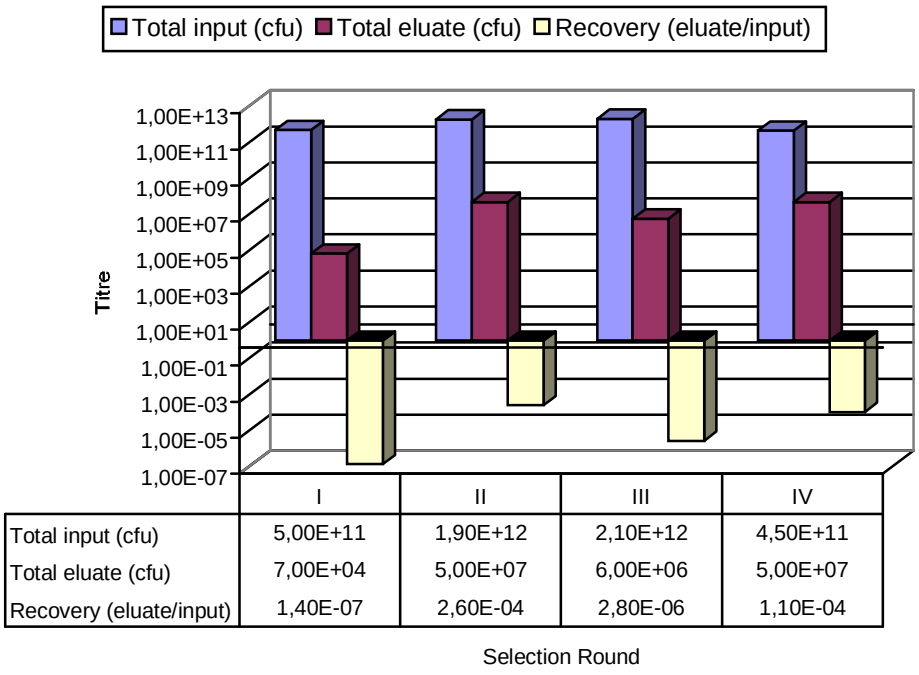


Figure 2.20: Ti(IV) ReactiBind selection

An enrichment of a factor 1000 was quite encouraging to find out about the sequences involved for the specificity of binding to the affinity matrix. Five clones were evaluated by sequencing.

Table 2.13: Sequences obtained from Ti(IV) ReactiBind selection

Clone number	Insert sequence	Frequency
TiRBIV#1	**H** R **H** **H** R P **H** E **H** S **H** R V T P	7
	deletions	3

Surprisingly, all insert containing clones displayed the same sequence which was selected with numerous of the other pannings on transition metals. Therefore, a control panning was performed to verify the specificity of binding.

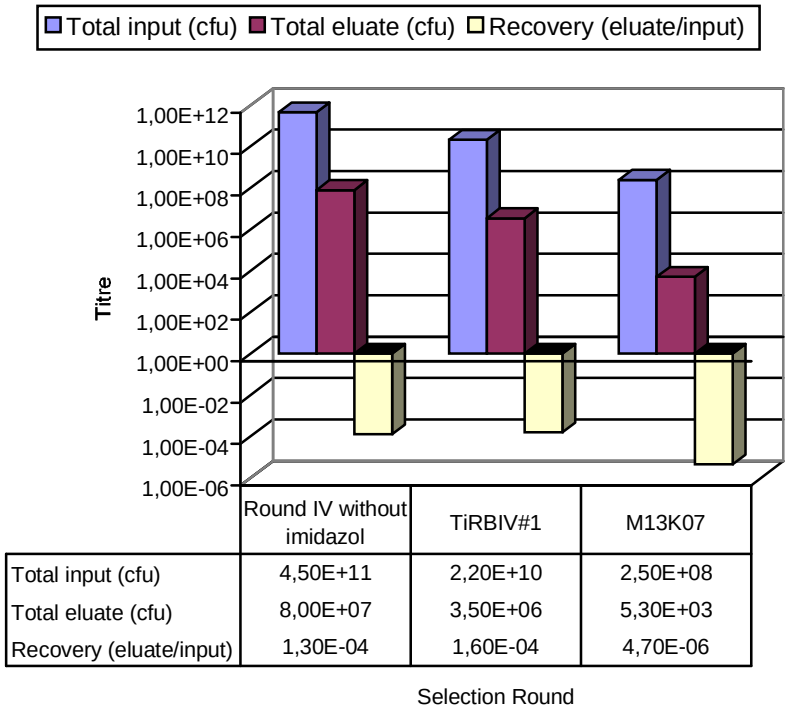
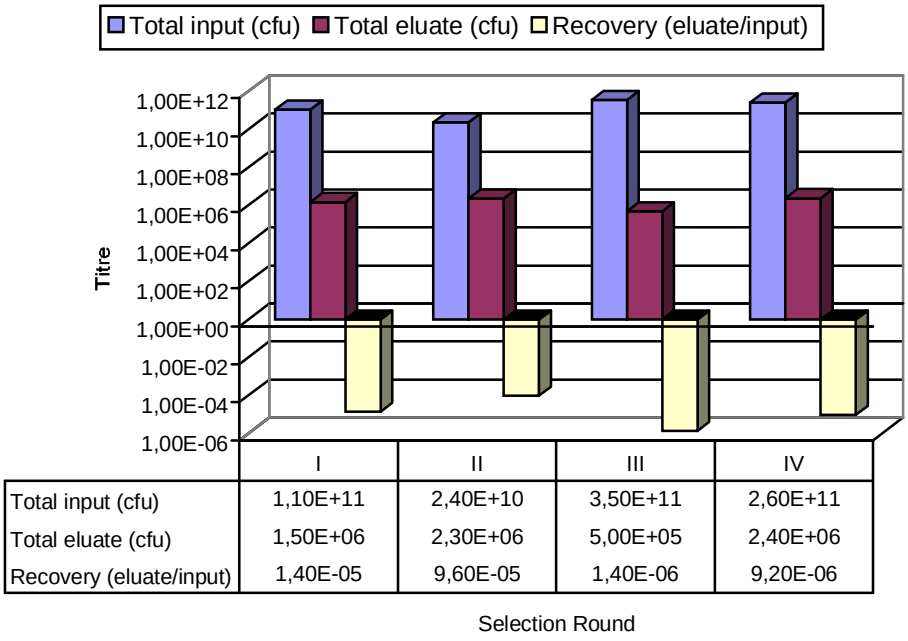


Figure 2.21: Ti(IV) ReactiBind control

There seems to be a sufficient difference between the control and the clone TiRBIV#1. Interestingly, the results remain the same with or without imidazole used in the washing buffer. This should account for a different mode of binding compared to the other transition metals.

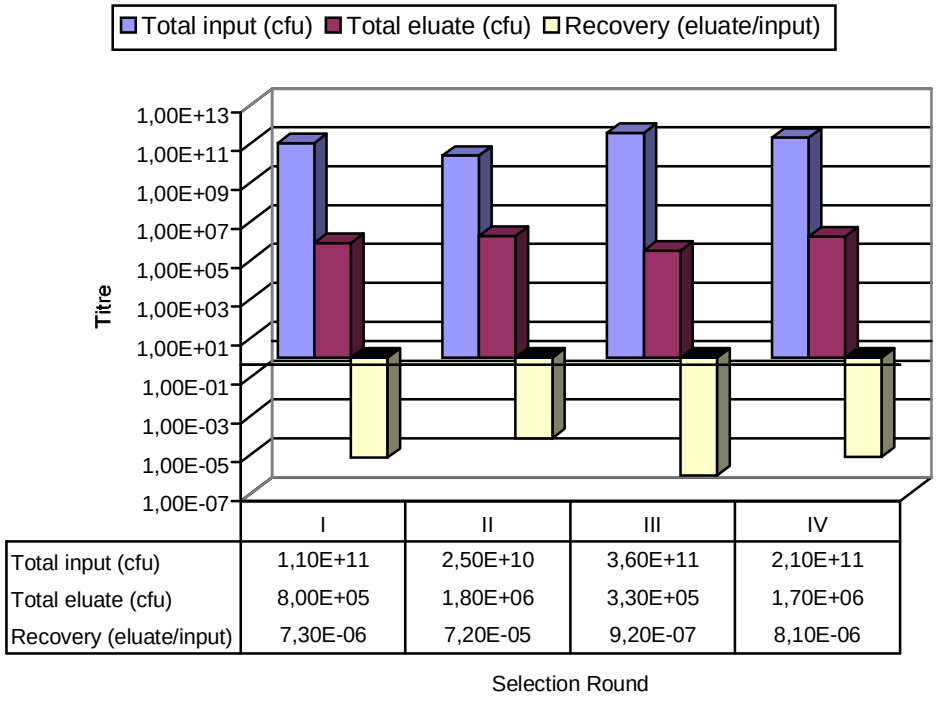
2.2.4 Iron(III) and Aluminium(III) selection

In order to select for variants which bind to the hard Lewis acids which bind even in the presence of imidazole the following selection procedures were used. Otherwise, the conditions are comparable to those applied to SpinZyme including the elution with 50mM EDTA. Similar results were obtained by the selection on iron(III) ReactiBind.



	I	II	III	IV
Total input (cfu)	1,10E+11	2,40E+10	3,50E+11	2,60E+11
Total eluate (cfu)	1,50E+06	2,30E+06	5,00E+05	2,40E+06
Recovery (eluate/input)	1,40E-05	9,60E-05	1,40E-06	9,20E-06

Figure 2.22: Al(III) ReactiBind selection



	I	II	III	IV
Total input (cfu)	1,10E+11	2,50E+10	3,60E+11	2,10E+11
Total eluate (cfu)	8,00E+05	1,80E+06	3,30E+05	1,70E+06
Recovery (eluate/input)	7,30E-06	7,20E-05	9,20E-07	8,10E-06

Figure 2.23: Fe(III) ReactiBind selection

Both selection experiments on iron(III) and aluminium(III) did not display a significant enrichment over the panning rounds. From the previous experience that deletions accumulate over the rounds under these conditions, five clones from Al(III) and Fe(III) were picked from the 3rd additional the five clones from the 4th round for sequencing.

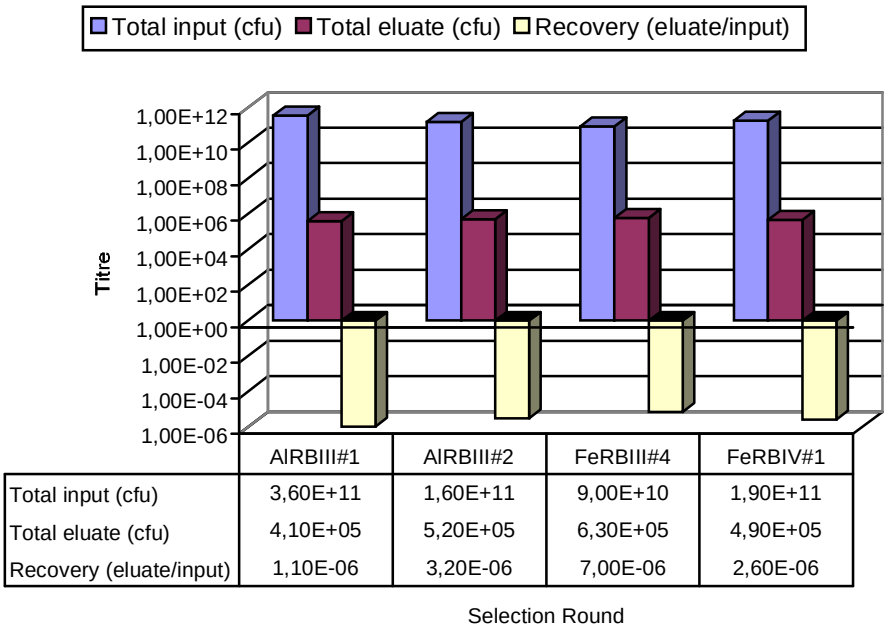
Table 2.14: Sequences obtained from Fe(III) ReactiBind selection

Clone number	Insert sequence	Frequency
FeRBIII#4	I S L S N **H** R M G W **H** **H** N Y S	1
FeRBIII#5	Q L P A T T **H** F R A P L G	1
FeRBIII	deletions	3
FeRBIV#1	Q L P A T T **H** F R A P L G	3
FeRBIV	deletions	2

Table 2.15: Sequences obtained from Al(III) ReactiBind selection

Clone number	Insert sequence	Frequency
AlRBIII#1	R D R V L **H** **H** A R V T S L **H** A	1
AlRBIII#2	P P Q K Q **H** A T F W P **H** F **H** N	1
AlRBIII	deletions	3
AlRBIV	deletions	5

Unlike the SpinZyme clones for these metal ions, only histidine can be associated with binding. The affinity may be very low since many deletions have accumulated. Therefore some control pannings were performed with some of the selected clones. In contrast to the previous selections, the MOPS buffer contained .5M NaCl.



	AlRBIII#1	AlRBIII#2	FeRBIII#4	FeRBIV#1
Total input (cfu)	3,60E+11	1,60E+11	9,00E+10	1,90E+11
Total eluate (cfu)	4,10E+05	5,20E+05	6,30E+05	4,90E+05
Recovery (eluate/input)	1,10E-06	3,20E-06	7,00E-06	2,60E-06

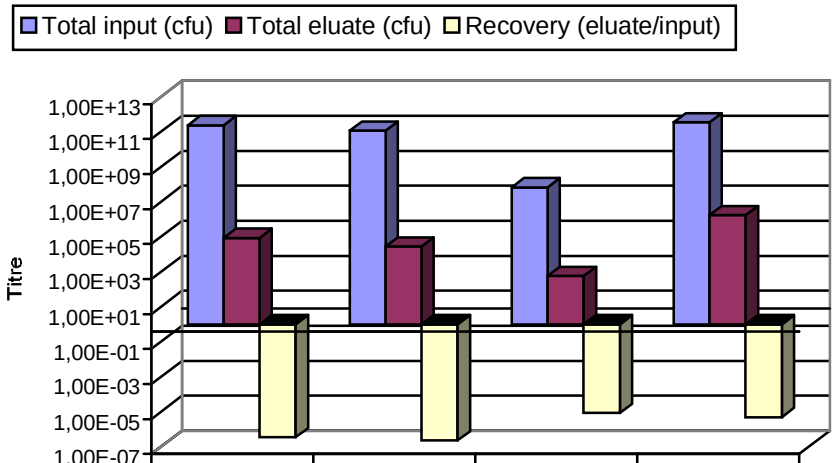
Selection Round

Figure 2.24: Al(III)+Fe(III) ReactiBind controls

The extremely low recovery titres suggest that under the conditions tested no specific binding occurs at all.

2.2.5 Fast lane panning

In order to develop a protocol which allowed an even faster selection and amplification of a phage library, the so-called “fast lane” panning was tested. All the panning protocols remained rather the same, utilising the same buffers as before for the transition metals. The major difference was the direct panning of the supernatants from overnight cultures of *E. coli* producing the phage progeny, as well as reducing the incubation times. This allowed one complete round of selection to be carried through at a single day. As a panning target, nickel(II) ReactiBind was chosen. Panning was essentially the same as for the transition metal ions. Elution was accomplished by to different approaches. FLA samples were eluted by a phosphate buffer at pH4 and FLB sample by the conventional imidazole elution buffer, each for 15 minutes at room temperature.

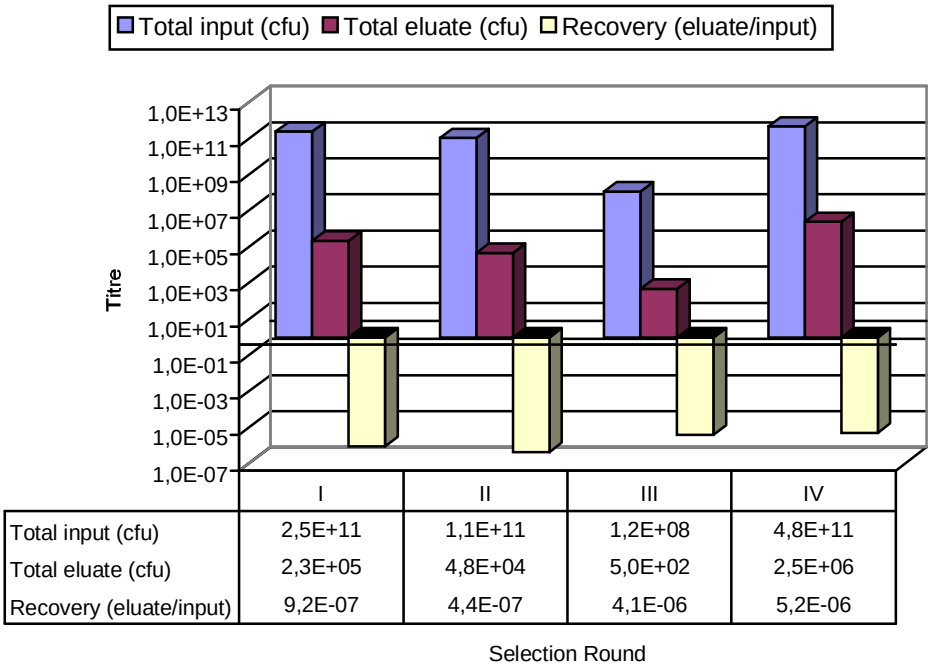


	I	II	III	IV
Total input (cfu)	2,50E+11	1,20E+11	6,80E+07	3,60E+11
Total eluate (cfu)	9,00E+04	2,80E+04	6,00E+02	1,80E+06
Recovery (eluate/input)	3,60E-07	2,30E-07	8,80E-06	5,00E-06

Selection Round

Figure 2.25: Fast Lane A selection

The parallel panned phage population FLB yielded almost the same results though different elution conditions were applied.



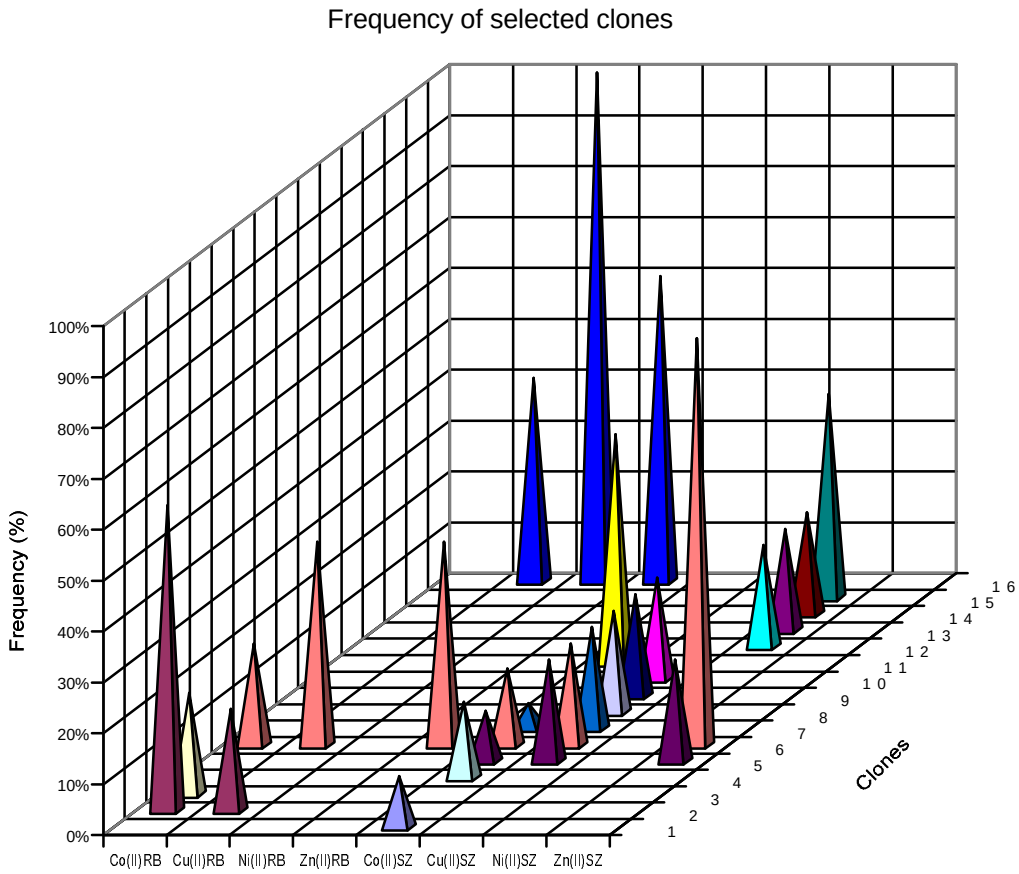
	I	II	III	IV
Total input (cfu)	2,5E+11	1,1E+11	1,2E+08	4,8E+11
Total eluate (cfu)	2,3E+05	4,8E+04	5,0E+02	2,5E+06
Recovery (eluate/input)	9,2E-07	4,4E-07	4,1E-06	5,2E-06

Figure 2.26: Fast Lane B selection

Due to the extremely poor enrichment of a factor 10, and the previous experiences with such figures, no further investigations were made at this point.

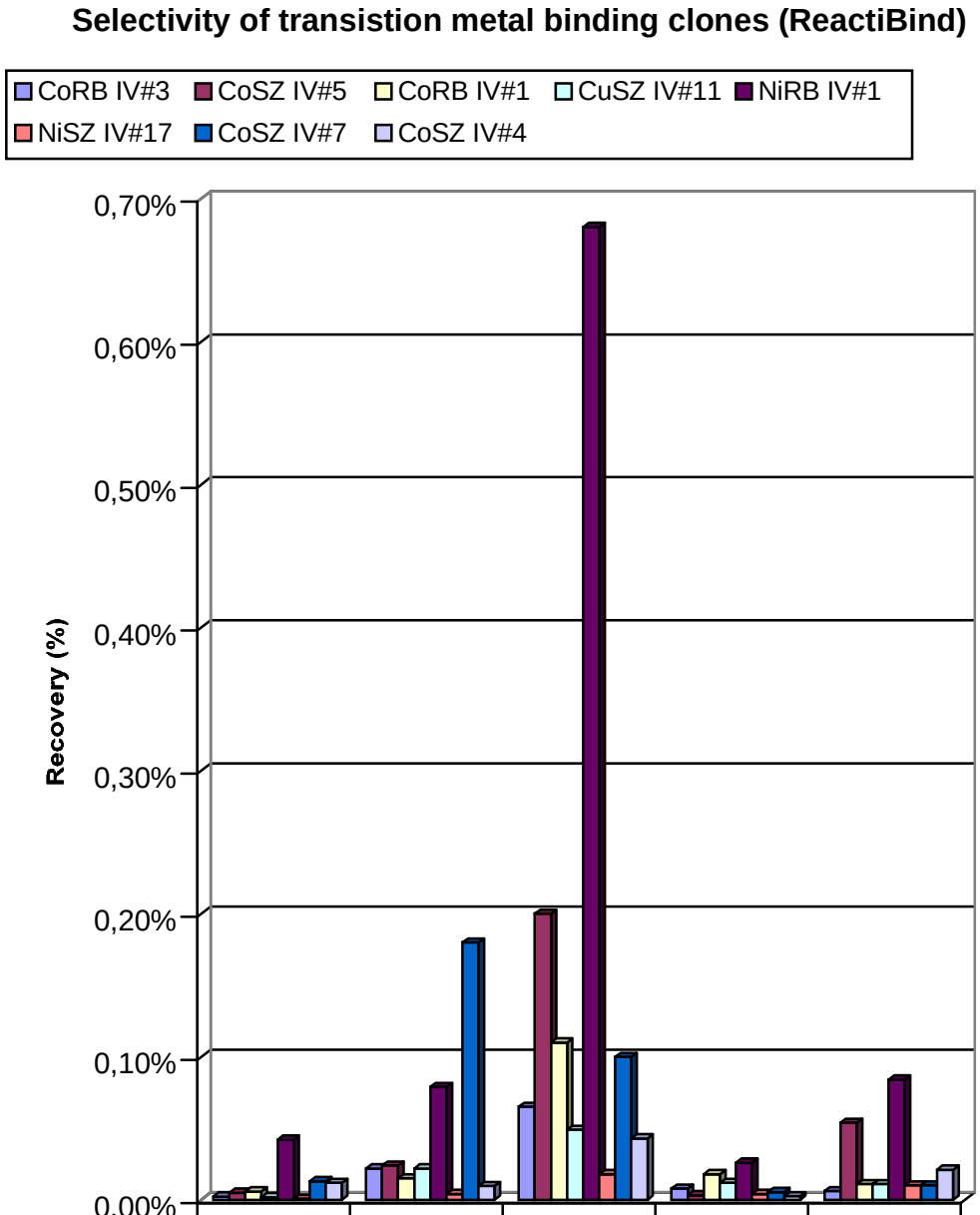
2.3 Cross-reactivity

For several clones did reappear during the panning on different metal ions and support materials, it is important to know about the preferences of these for some of the affinity materials. Therefore, cross-reactivity tests were made to assess the specificity of clones being either unique or prevalent in the selection on one of the affinity materials. Unfortunately, some clones were lost and could not be tested at the end of this study. Representative clones were selected on the transition metals immobilised on the two different affinity materials SpinZyme and ReactiBind. The recoveries from each of the individual selections are listed below. Incubation of phage were performed with 3%BSA in wash-PBS. SpinZyme and ReactiBind was rinsed with wash-PBS containing 20mM imidazole. The same conditions were chosen for Fe(III) except for the imidazole in the washing buffer.



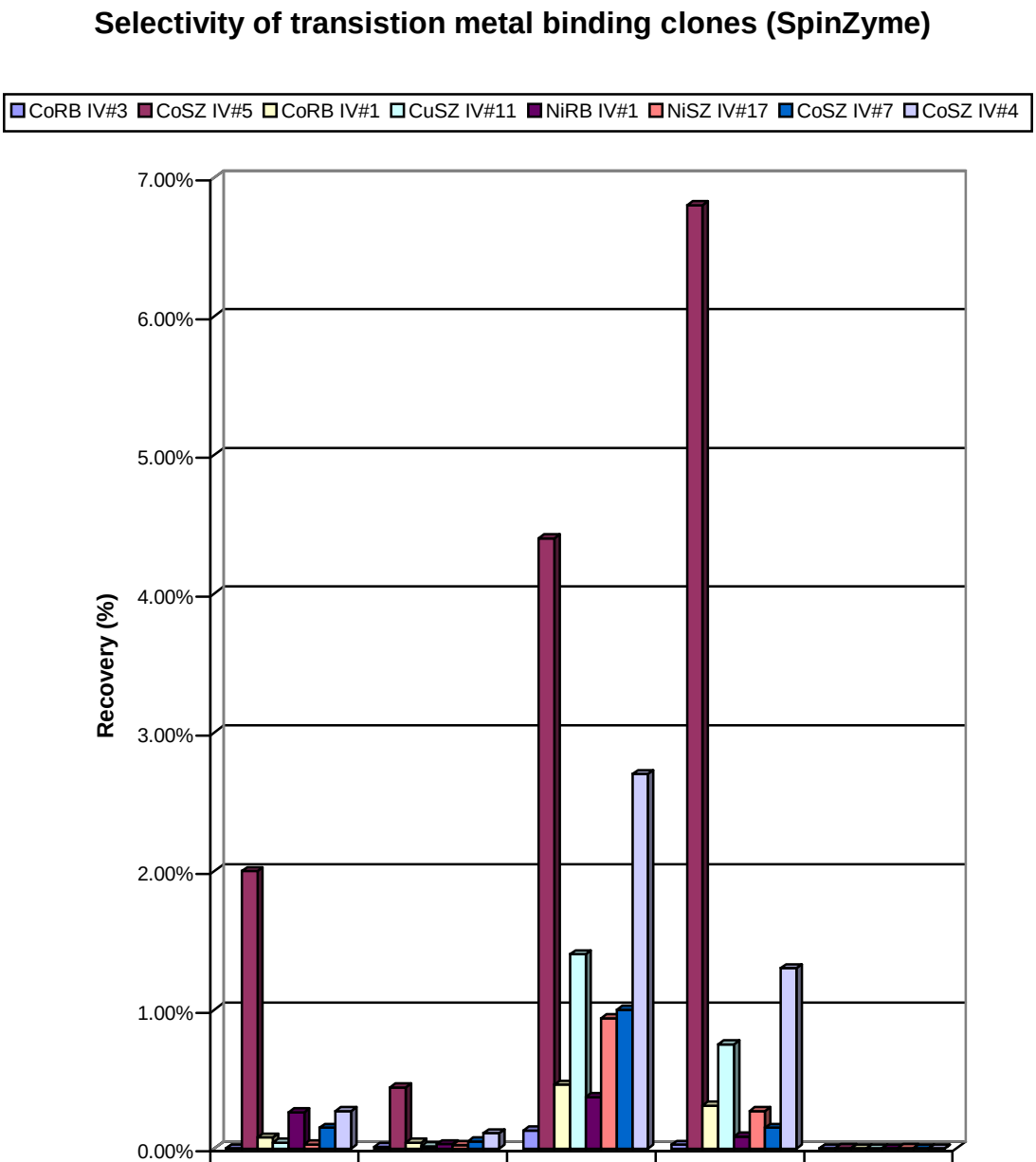
Row	Clone	Sequence	Row	Clone	Sequence
1	Deletions	none	9	CuSZIV#14	HRSWTSPHNHPHTHH
2	CoRBIV#1	AHQQTHHYFTHHLNY	10	CuSZIV#15	KHHLHHEHAYPTLKN
3	CoRBIV#3	VAHHWWHDGYKHPLN	11	CoSZIV#1	THSTHPASHHRHKHT
4	CoSZIV#4	ALPRSSPHHHHLPHR	12	NiSZIV#16	LDHTYRAHSKVHHHH
5	CoSZIV#5	MGSNHMHHHHFPHLP	13	NiSZIV#17	APSHHTHSHHLTQMR
6	CoSZIV#7	HRHHRPHGDTHRVTP	14	NiSZIV#18	YHTSIHHHHPVDHLA
7	CoSZIV#11	PHQGYHKATHHHWSP	15	NiSZIV#20	AYPHPHSNSHLIHSH
8	CuSZIV#11	AHPHRHHSDSMLVTH	16	NiRBIV#1	HHHHSYMSSIPSTAW

Figure 2.27: Frequency of clones in all selections



	Co(II)-RB	Zn(II)-RB	Ni(II)-RB	Cu(II)-RB	Fe(III)-RB
CoRB IV#3	2,30E-05	2,20E-04	6,50E-04	7,80E-05	6,10E-05
CoSZ IV#5	5,00E-05	2,40E-04	2,00E-03	3,20E-05	5,40E-04
CoRB IV#1	5,90E-05	1,50E-04	1,10E-03	1,80E-04	1,10E-04
CuSZ IV#11	1,80E-05	2,20E-04	4,90E-04	1,20E-04	1,10E-04
NiRB IV#1	4,20E-04	7,90E-04	6,80E-03	2,60E-04	8,40E-04
NiSZ IV#17	7,00E-06	4,00E-05	1,80E-04	4,00E-05	1,00E-04
CoSZ IV#7	1,30E-04	1,80E-03	1,00E-03	5,50E-05	1,00E-04
CoSZ IV#4	1,20E-04	9,70E-05	4,30E-04	2,40E-05	2,10E-04

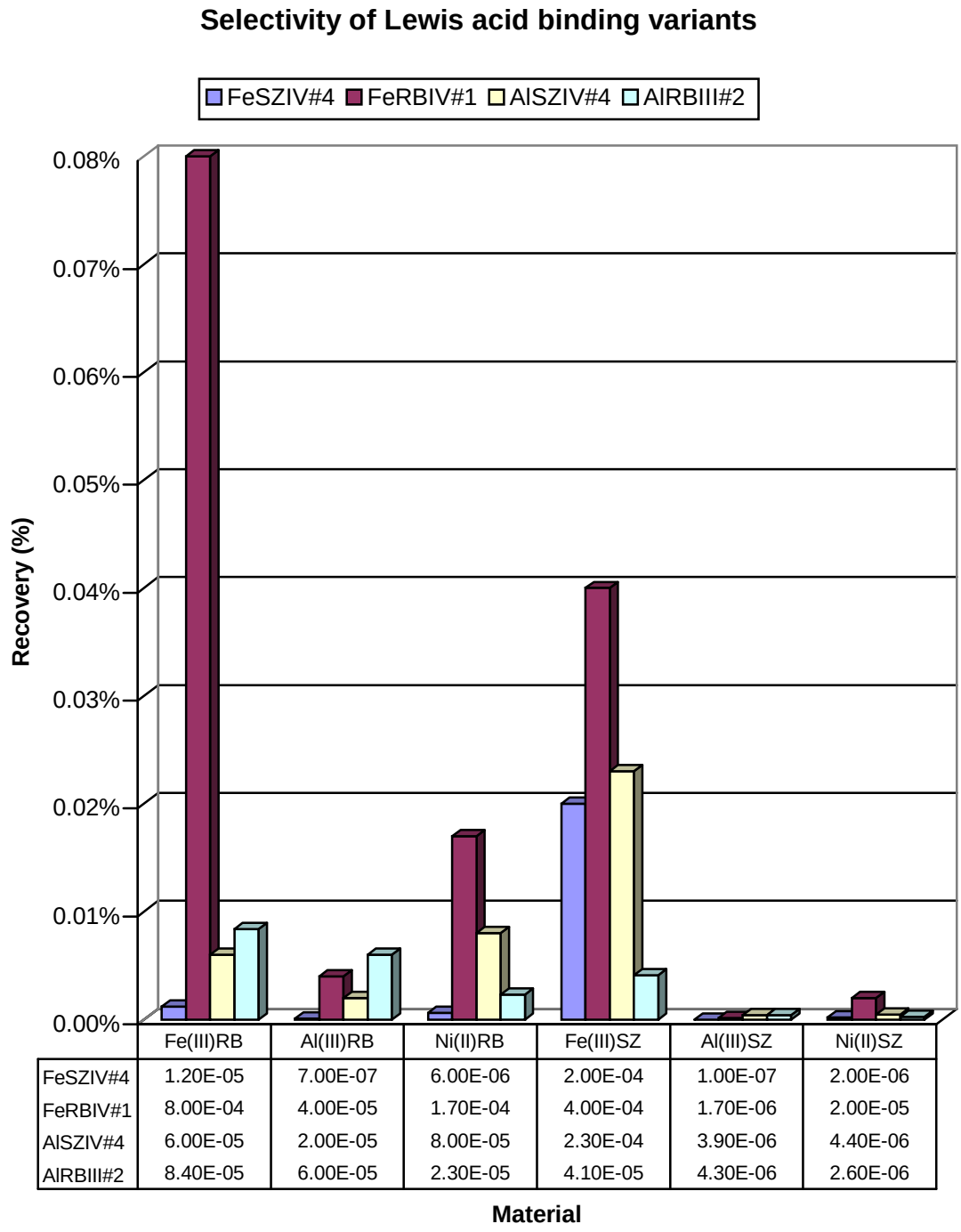
Figure 3.28: Selectivity of transition metals on ReactiBind for different clones



	Co(II)-SZ	Zn(II)-SZ	Ni(II)-SZ	Cu(II)-SZ	Fe(III)-SZ
CoRB IV#3	8.30E-06	1.30E-04	1.30E-03	2.70E-04	6.10E-06
CoSZ IV#5	2.00E-02	4.40E-03	4.40E-02	6.80E-02	2.20E-05
CoRB IV#1	8.00E-04	4.20E-04	4.60E-03	3.10E-03	5.00E-06
CuSZ IV#11	4.30E-04	1.50E-04	1.40E-02	7.50E-03	2.20E-06
NiRB IV#1	2.60E-03	3.10E-04	3.70E-03	8.70E-04	5.00E-06
NiSZ IV#17	3.00E-04	2.40E-04	9.40E-03	2.70E-03	2.10E-05
CoSZ IV#7	1.50E-03	5.00E-04	1.00E-02	1.50E-03	1.00E-05
CoSZ IV#4	2.70E-03	1.10E-03	2.70E-02	1.30E-02	3.40E-06

Figure 2.29: Selectivity of transition metals on SpinZyme for different clones

Cross-reactivity of the hard Lewis acids in SpinZyme and ReactiBind. Sample were treated as above, using wash-MOPS-T pH7.4 instead of wash-PBS-T with imidazole.



	Fe(III)RB	Al(III)RB	Ni(II)RB	Fe(III)SZ	Al(III)SZ	Ni(II)SZ
FeSZIV#4	1.20E-05	7.00E-07	6.00E-06	2.00E-04	1.00E-07	2.00E-06
FeRBIV#1	8.00E-04	4.00E-05	1.70E-04	4.00E-04	1.70E-06	2.00E-05
AlSZIV#4	6.00E-05	2.00E-05	8.00E-05	2.30E-04	3.90E-06	4.40E-06
AlRBIII#2	8.40E-05	6.00E-05	2.30E-05	4.10E-05	4.30E-06	2.60E-06

Figure 2.30: Selectivity of immobilised lewis acids for different clones

2.4 Applications for IMAC phage

For new applications it is important to evaluate the affinity of a transition metal binding phage to different materials under varying conditions. It may be useful constructing a helper phage with an IMAC tag, allowing a purification of packaged phagemids by IMAC. Fusions of the affinity sequence with other proteins may serve as an affinity tag for affinity purification. Materials such as Chelating Sepharose FF, Ni-NTA and Talon Metal Affinity Resin are readily available and mainly applied for the purification of His6-tagged proteins.

2.4.1 Phage preparation by IMAC

Chelating Sepharose Fast Flow

Two approaches were made using 10µl and 20µl of Co(II) charged Chelating Sepharose FF (CoChS), respectively. Due to the relative poor recovery of phage, the experiment was repeated two days later with the stored supernatant using 100µl of CoChS. During the incubation of the 10ml supernatant, a bleaching of the sepharose was observed. Elution was performed with 2x200µl .5M imidazole PBS.

Talon Metal Affinity Resin

As the Talon Metal Affinity Resin is a tetradentate chelator complexed with cobalt(II), it is more resistant under various conditions against metal leeching. Only a preliminary experiment was set up to check the suitability of the material for a phage preparation.The recovery seems to be better compared to the previous approach with Chelating Sepharose FF.

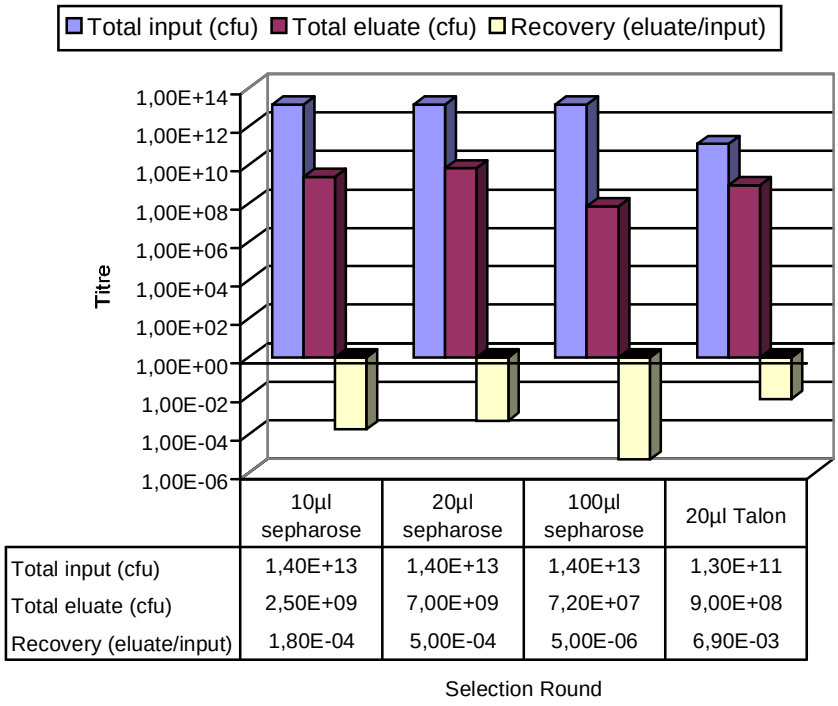


Figure 2.31: Bacteriophage preparation from bacterial culture supernatants by metal affinity resins.

2.4.2 Purification of pIII fusions

For the purification of the pIII fusions, the filamentous phage need to be disrupted. This can be achieved by the application of strong ionic detergents such as SDS or the use of chaotropic salts as guanidinium hydrochloride or urea. As IMAC is known to be best compatible with urea, different concentrations were assayed to accomplish the task. The C-terminus of the pIII protein is hydrophobic and connects to the phage coat. To elute this protein from the chromatography material under non-denaturing conditions completely, SDS could be used in an additional washing step. Two materials were tested for the suitability of purification.

2.4.2.1 Cu(II) SpinZyme

Phage from clone CoSZIV#5 were subjected to denaturation in various amounts of urea. The samples were purified on cobalt(II) SpinZyme affinity separations units and eluted by an acidic phosphate buffer. 15µl aliquots corresponding to ¼ of the sample were loaded on a 12.5% SDS polyacrylamide gel. For the visualisation of the purified protein, a western transfer to a nitrocellulose membrane was made. The membrane was incubated with anti-pIII-mAb and anti-mouse-Ig_1-HRP conjugate and developed with the metal enhanced DAB staining method. Unfortunately, no pIII protein other than the one originating from the input control sample became visible.

2.4.2.2 Chelating Sepharose Fast Flow

It seemed that the commercially available Chelating Sepharose FF is more suitable for the task of protein purification, since many protocols are already available using just this chromatography material. Varying amounts of urea were added to NiSZIV#18 and NiRBIV#1. A small column was prepared, retaining the previously added resin. Elution was achieved with imidazole. 2/5 of each eluted sample was loaded on a 12.5% SDS polyacrylamide gel for electrophoresis. The gel was stained with coomassie-blue and destained to reveal the proteins contained in the different lanes. The minor coat protein was not directly visible by this method. Therefore, a western blot with anti-pIII-mAb and DAB stain was performed as described above. Besides the coomassie-stain which was transferred to the membrane, no additional staining became visible.

In order to evaluate whether the pIII protein remains on the resin even after elution with imidazole due to its hydrophobicity, the purification procedure was repeated. The conditions remained the same, only twice as much sample was applied. The elution with .5M imidazole PBS was followed by an additional step with .5M imidazole PBS with 1% SDS. The western blot did not produce any different results from the previous approach.

2.4.2.3 Talon Affinity Resin

As the supplier of the Talon Affinity Resin claims the extreme stability of this resin to numerous chemicals during the purification, this material was chosen for the denaturing purification using high molarities of urea.. Elution was achieved by washes with .5 imidazole PBS to be followed by .5M imidazole PBS 1% SDS. Half of these eluted samples were separated by a 12.5% SDS-PAGE and subseqeuntly transferred to a nitrocellulose membrane. The western blot with the anti-pIII-mAb revealed that most of the protein passed through the resin without binding. Extremely faint signals may be seen for the elution samples.

2.4.2.4 Ni(II)-NTA agarose

Just like the Talon resin, Ni(II)-NTA is thought to be very resistant against leeching of the metal ion in various chemical environments.

Urea variation

Different urea concentrations, 2M, 4M and 6M were assayed to both accomplish denaturation and allow purification of pIII derived from CoSZIV#7 phage. Washing was done with 1ml wash-PBS. The elution was achieved .5M imidazole and .5M imidazole 1% SDS. Each aliquot was loaded on two separate 12.5% SDS-polyacrylamide gels. After electrophoresis one gel was stained by coomassie blue whereas the other was transferred to a nitrocellulose membrane.

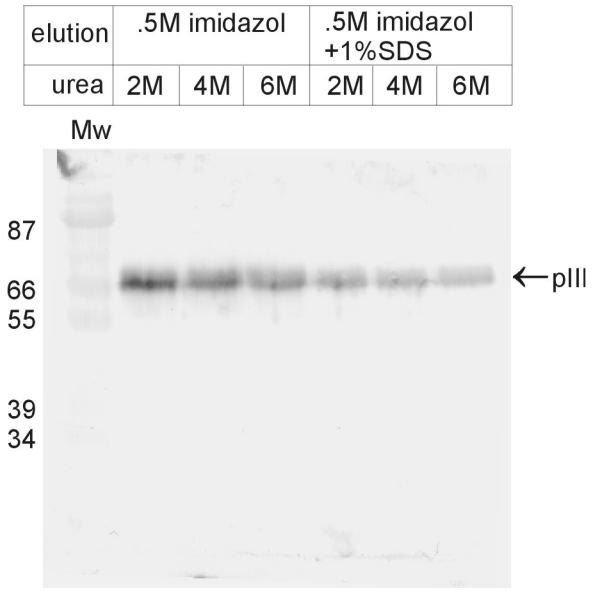


Figure 2.32: Western blot of the Ni(II)-NTA agarose chromatography with CoSZIV#7. Mw denotes the molecular weight standard

The western blot was able to detect the pIII protein in all lanes. The signal did not depend on the urea concentration used for denaturation. However, it showed that half of the pIII protein remained on the resin without the addition of detergent. The coomassie stain did not reveal a band for the pIII protein, because the protein concentration is too low.

Optimisation

Only 4M urea were used for the denaturation of the phage. Four different samples were prepared with respect to their washing conditions during the chromatography.

a) silver stain

elution	.5 M imidazol				.5M imidazol + 1%SDS			
wash	1ml		4ml		1ml		4ml	
imidazol	-	+	-	+	-	+	-	+

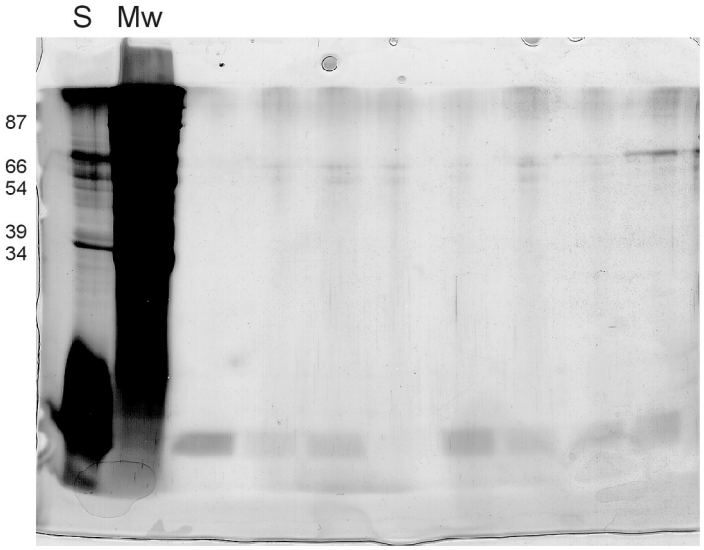


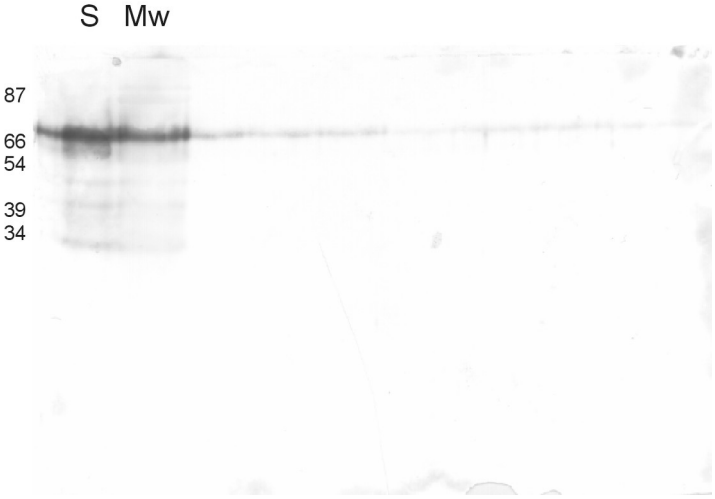
Figure 2.33: SDS-PAGE from Ni-NTA agarose chromatography of CoSZIV#7.

S input sample

Mw molecular weight standard

b) anti-pIII antibody DAB stain

elution	.5 M imidazol				.5M imidazol + 1%SDS			
wash	1ml		4ml		1ml		4ml	
imidazol	-	+	-	+	-	+	-	+



Sample	Denaturing wash	Native wash
I	1ml 4M urea wash-PBS	1ml wash-PBS
II	1ml 4M urea wash-PBS 10mM imidazole	1ml wash-PBS 10mM imidazole
III	4ml 4M urea wash-PBS	1ml wash-PBS
IV	4ml 4M urea wash-PBS 10mM imidazole	1ml wash-PBS 10mM imidazole

Samples were eluted with 100µl .5M imidazole pH7.4 and then with 100µl .5M imidazole pH7.4 1% SDS. Two separate 12.5% SDS-polyacrylamide gels were loaded with each 15µl of the 100µl elution samples for electrophoresis. The intact phage served as a control. One gel was silver-stained, whereas the other was transferred to a membrane for a western blot.

The pIII signals from the western blot in the different samples were a faint, but almost all of them had the same intensity. In contrast, the silver stain revealed that the contaminating pVIII band was less in the samples treated with imidazole and even less in those washed more extensively than the others. In total, the recovery of the pIII protein from the initial concentration was about 10%.

2.4.2.5 Fe(III)-NTA agarose

From the previous purification experiments with the transition metal ion binding variants it became obvious that materials as Chelating Sepharose FF and even the tetradentate Talon Affinity Resin were not stable or selective enough to purify the pIII fusion proteins. For this task NTA was chosen to be the ideal support material for iron(III). As NTA is shipped readily chelated with nickel(II), it has to be stripped prior to complexing it with iron(III). For analysis, 1/10 of the eluted samples were loaded on a 12.5% SDS-polyacrylamide gel to be separated by electrophoresis. A western transfer was done and detection with anti-pIIIImAb was achieve via anti-mouseIg_1-HRP conjugate by DAB staining.

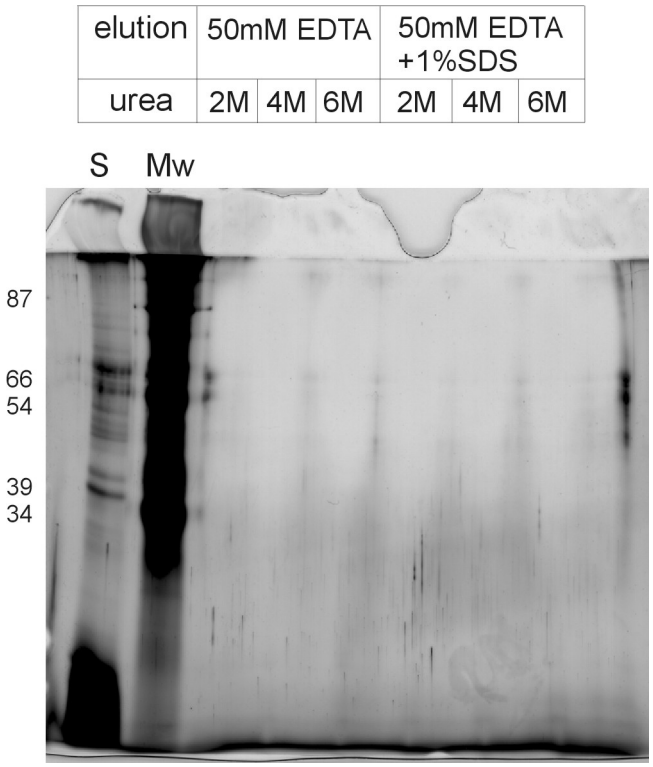


Figure 2.34: silver stain of Fe(III)-NTA agarose chromatography of FeSZIV#1 using different urea concentrations

S sample input

Mw molecular weight standard

The pIII fusions became faintly visible in all of the samples to a similar extent. This is almost comparable to the results obtained by the purification of CoSZIV#7 previously.

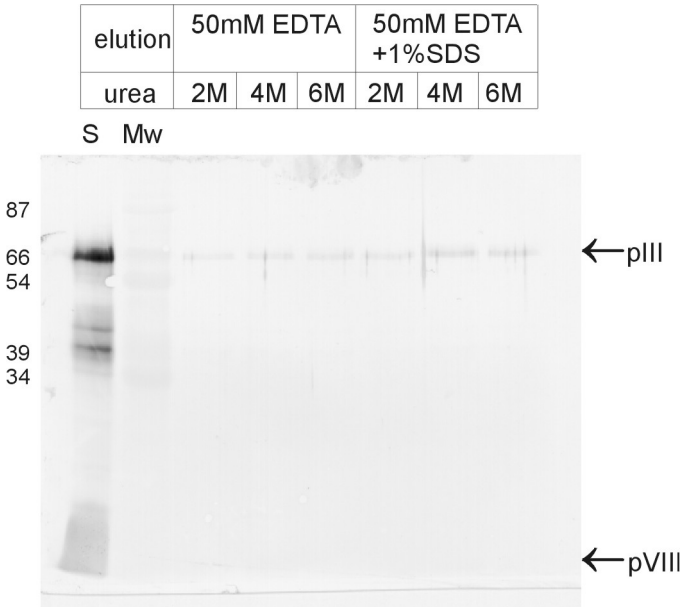


Figure 2.35: Western blot of Fe(III)-NTA agarose chromatography of FeSZIV#1 using different urea concentrations DAB stain with anti-pIII and anti M13 antibodies.

S sample input

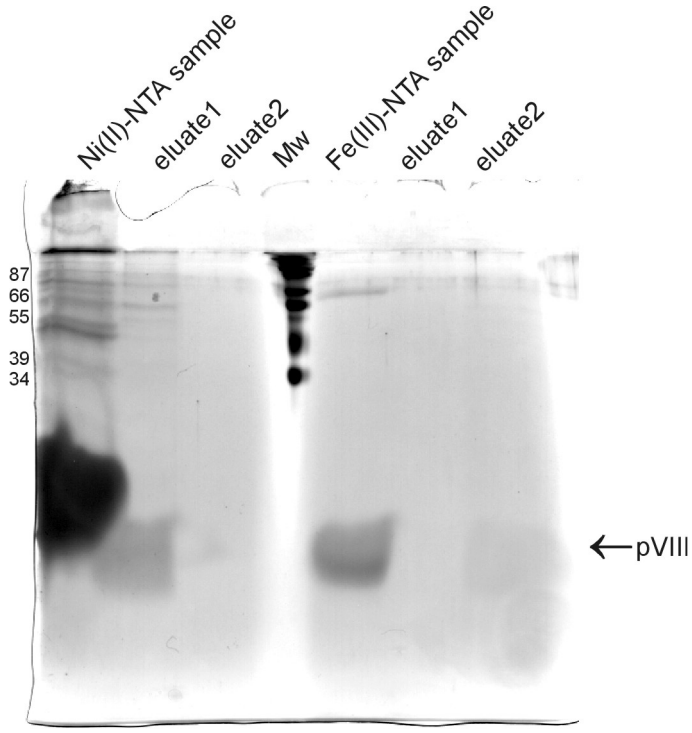
Mw molecular weight standard

Due to the very weak signals, the efficiency of the purification method cannot be estimated exactly. The blot was scanned and then incubated with the anti-M13-HRP conjugate directed against the major coat protein pVIII. The second DAB staining revealed that the initially great amount of pVIII protein cannot be detected in the eluted samples. This demonstrates a significant enrichment of the pIII fusion with this method.

2.4.2.6 Comparison of Fe(III) and Ni(II)-NTA agarose

In order to compare the usefulness of a Fe(III) binding sequence for protein purification, two chromatographies were performed in parallel. The best Ni(II)-SpinZyme binding variant CoSZIV#5 was chosen for Ni(II)-NTA and FeSZIV#1 for the Fe(III)-NTA purification. To make sure that the phage are disrupted and the fusion peptide of pIII cannot form a structure, 6.8M urea was used for denaturation. The wash was performed with 8M urea containing buffer. Elution was done twice with imidazole for Ni(II)-NTA and EDTA for Fe(III)-NTA. 15µl of 100µl eluate were loaded on two 12.5% SDS-polyacrylamide gels. One was stained with coomassie, the other was transferred to nitrocellulose for a western blot. The blot was developed by consecutive DAB staining with anti-pIII+anti-mouseIg_1-HRP and anti-M13-HRP. It clearly demonstrates that both metal ion binding have similar recoveries under the conditions tested. Judging from the amount of input pIII, about 15% were recovered from the chromatography.

A) Silver stain



B) Anti-pIII antibody DAB stain

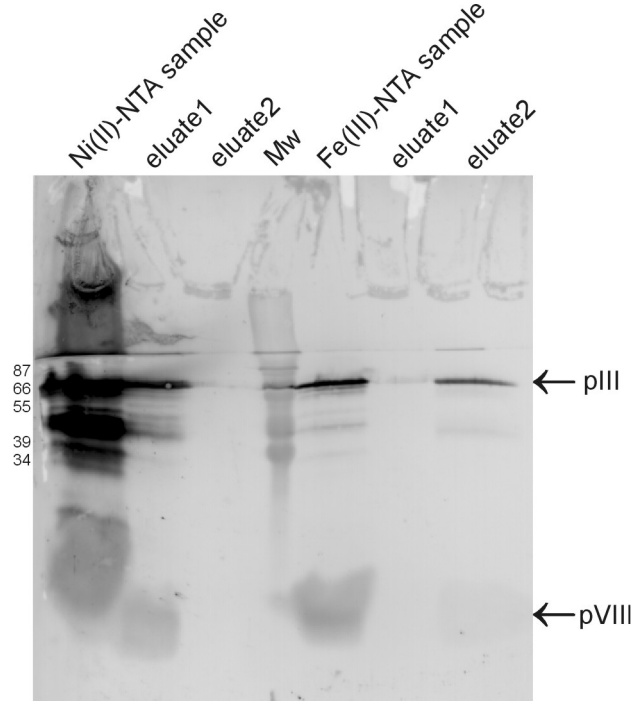


Figure 2.36: comparison of Ni(II)-NTA and Fe(III) NTA agarose chromatography.

A) coomassie stain of SDS- polyacrylamide gel

B) DAB stain of western blot with anti-pIII and anti-pVIII antibodies.

2.5 Characterisation of iron(III) binding clone FeSZIV#1

As estimated from the previous control pannings of different Fe(III) SpinZyme binding clones identified, FeSZIV#1 can be considered as the best clone.

2.5.1 Binding properties

To evaluate the mode of binding of the iron(III) binding phage, several different buffers and competitors were applied to model selection experiments.

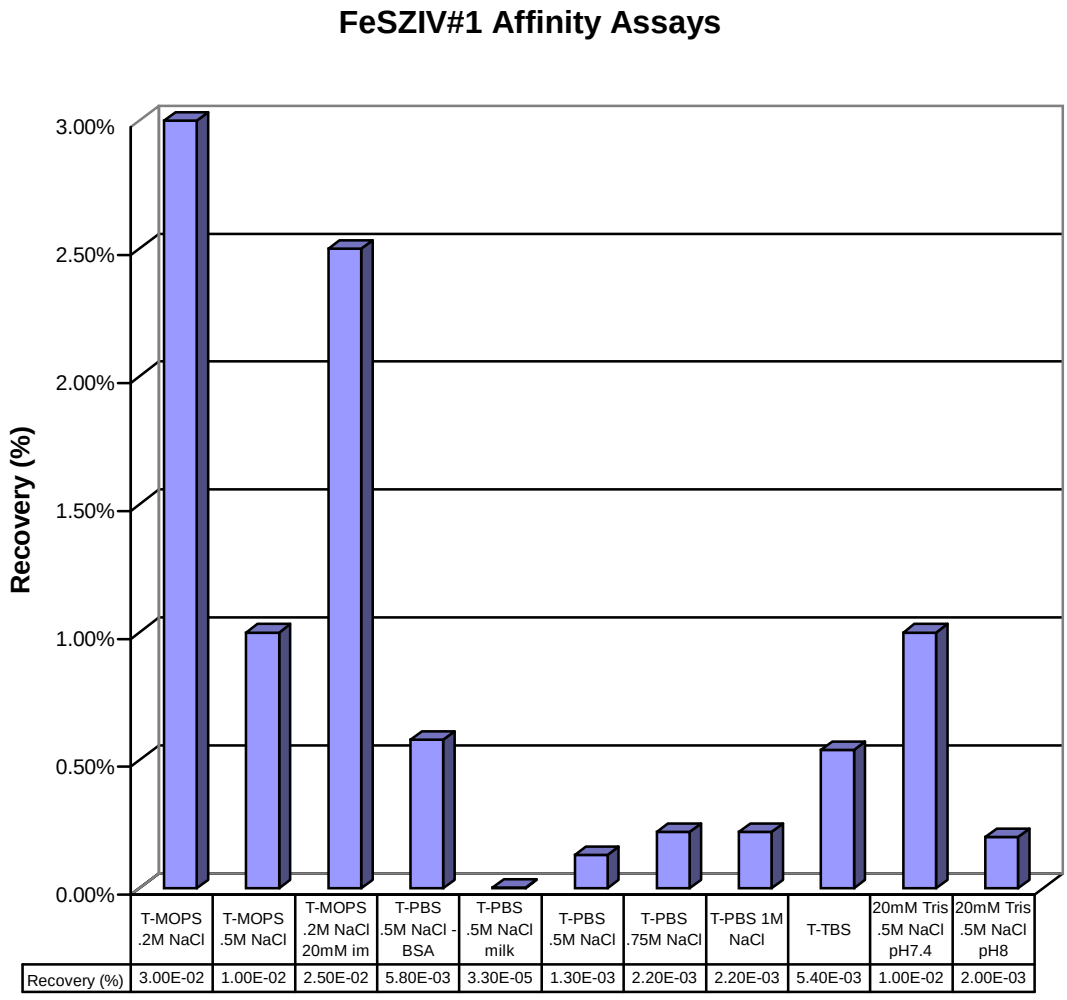
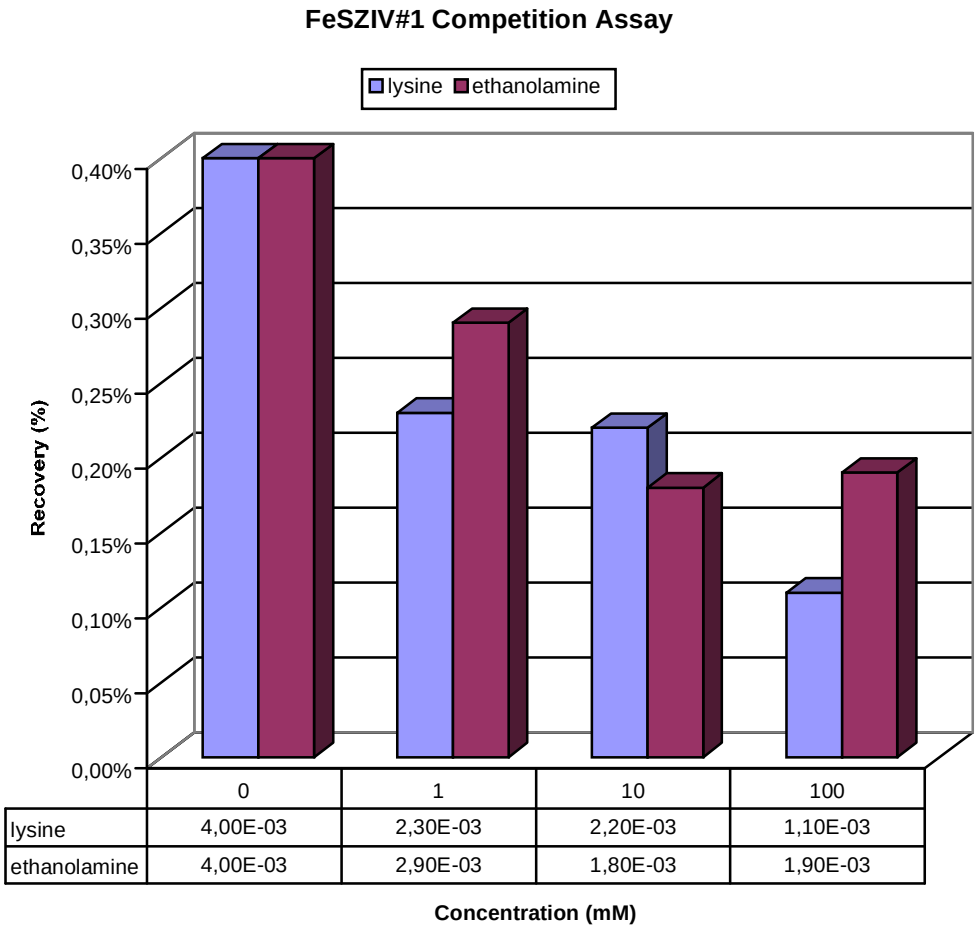


Figure 2.37: FeSZIV#1 affinity assays with varying buffer, salt and pH conditions.

In order to evaluate the role of lysine residues in binding to iron(III), different competitors were chosen in the next experiments. Blocking was performed with 3%BSA, 40mM MOPS .5M NaCl .05% Tween 20 pH7.4 served as a washing buffer.



	0	1	10	100
lysine	4,00E-03	2,30E-03	2,20E-03	1,10E-03
ethanolamine	4,00E-03	2,90E-03	1,80E-03	1,90E-03

Figure 2.38: FeSZIV#1 competition assay with primary amines lysine and ethanolamine.

2.5.2 Cross-reactivity

FeSZIV#1 was tested for cross-reactivity with the hard Lewis acids under the same conditions described above.

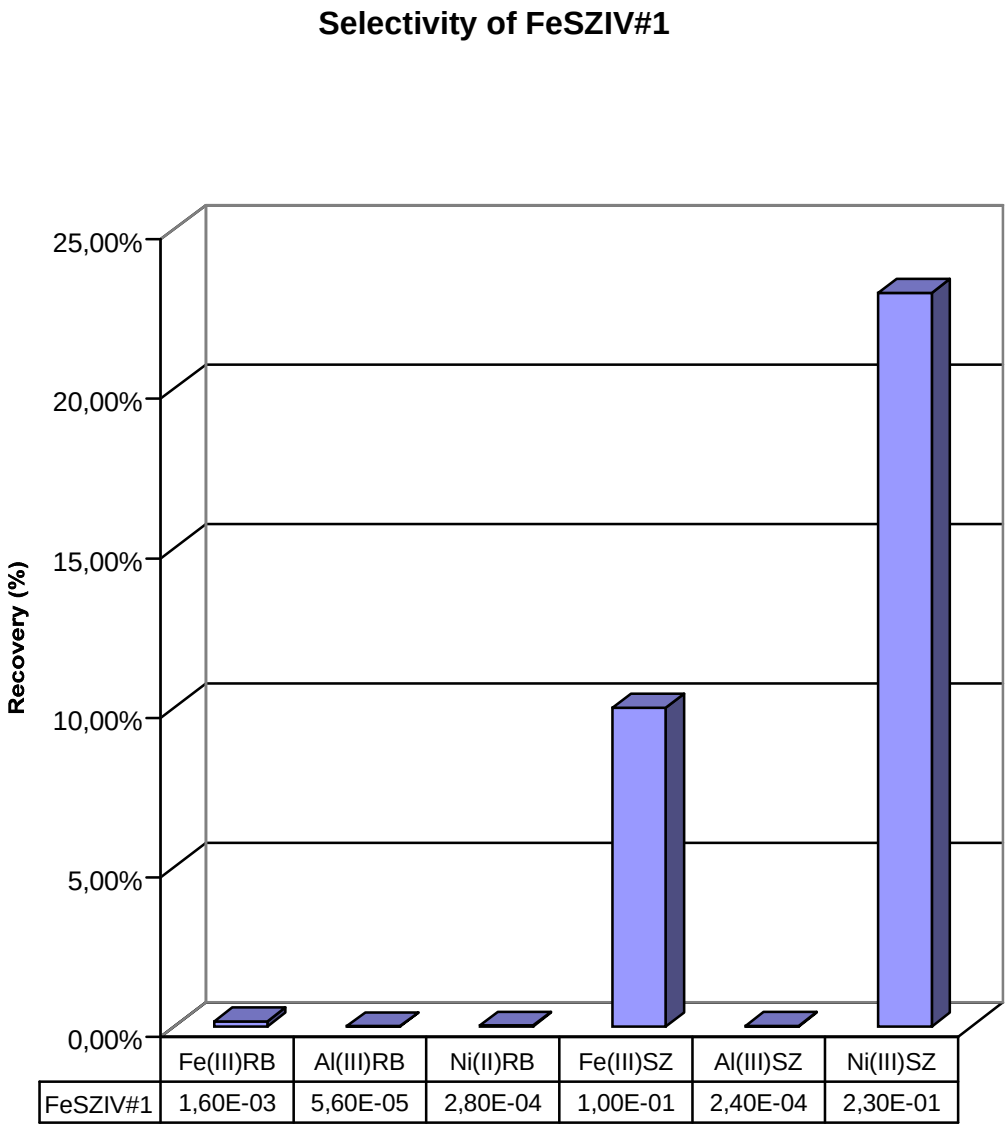


Figure 2.39: Selectivity of FeSZIV#1 for hard Lewis metal ions and nickel(II)

When it became apparent that this clone bound to Ni(II)-SpinZyme event better than to Fe(III)-SpinZyme, the binding to the other transition metals bound to SpinZyme was tested. The same conditions as for the transition metal cross-reactivity tests were applied, including the 20mM imidazole in the washing buffer. Though $5.2x10^8$ phage served as the input, no colonies were formed on the plates from all of the different

eluates. Thus below 1000 phage are contained in the eluates. The recoveries (eluate/input) are therefore below $2x10^{-6}$.

2.6 IMAC helper-phage construction

The helper phage M13K07 is a derivative of the filamentous phage M13 with an insertion of a kanamycin resistance gene cassette in the origin of replication. As no reliable sequence map exists of the helper phage itself, the parent sequence of the wild type phage was taken as a lead for the cloning procedure. By analysis with the VectorNTi 4.0 programme, most of the pIII sequence of M13 and M13LP67 is identical. BspMI, a type IIs restriction enzyme, serves best for subcloning the insert of M13LP67 into M13K07. It produces only two fragments in M13K07 and three in M13LP67. The 4 base overhangs created in one genome all differ from each other and are not palindromic, thus allowing only one assembly product upon re-ligation, resulting in the original phage.

M13K07 / BspMI	CoRBIV#1 / BspMI
Appr. 7500 bp	3972 bp
1163 bp	3104 bp
	1232 bp

The small fragments of 1163 bp from M13K07 and 1232 bp CoRBIV#1 correspond to each other and contain the N-terminus of the pIII gene.

The double stranded RF DNA was prepared from M13K07 and CoRBIV#1 from a 3ml overnight culture of infected *E. coli* JM103. The DNA was cleaved with BspMI and the resulting fragments were separated by agarose gel electrophoresis. After staining the DNA and visualisation with UV light, the 7.3 kb and 1.2 kb fragments from M13K07 and CoRBIV#1 respectively, were excised and eluted from the gel. Ligation with T4 ligase was performed over night at 4°C. An aliquot of the resulting ligation product was electroporated into electrocompetent XL-1 blue cells. After incubation at 37°C, 100µl of 1ml cell suspension was spread on an agar plate containing LB, 100µg/ml kanamycin and 20µg/ml tetracyclin. This resulted in about 50 cfu from which 6 individual clones were picked and grown as a 3ml LB Km 100 Tc20 overnight culture. Only two clones grew (#5 and #6), one of them to a density high enough for a DNA preparation. The prepared DNA from clone #6 was subjected to restriction analysis. As the M13K07 genome and the pIII gene from M13 is known to contain a unique BamHI restriction

site in the pIII gene, whereas the CoRBIV#1 does not, a digestion with this enzyme would clearly distinguish the original helper phage from the new clone. Therefore clone #6 and M13K07 were treated with BamHI and analysed by agarose gel electrophoresis. Clone #6 remained unmodified as M13K07 was linearised as a result of digestion. Henceforth the new helper phage clone #6 will be referred to as M13Co1.

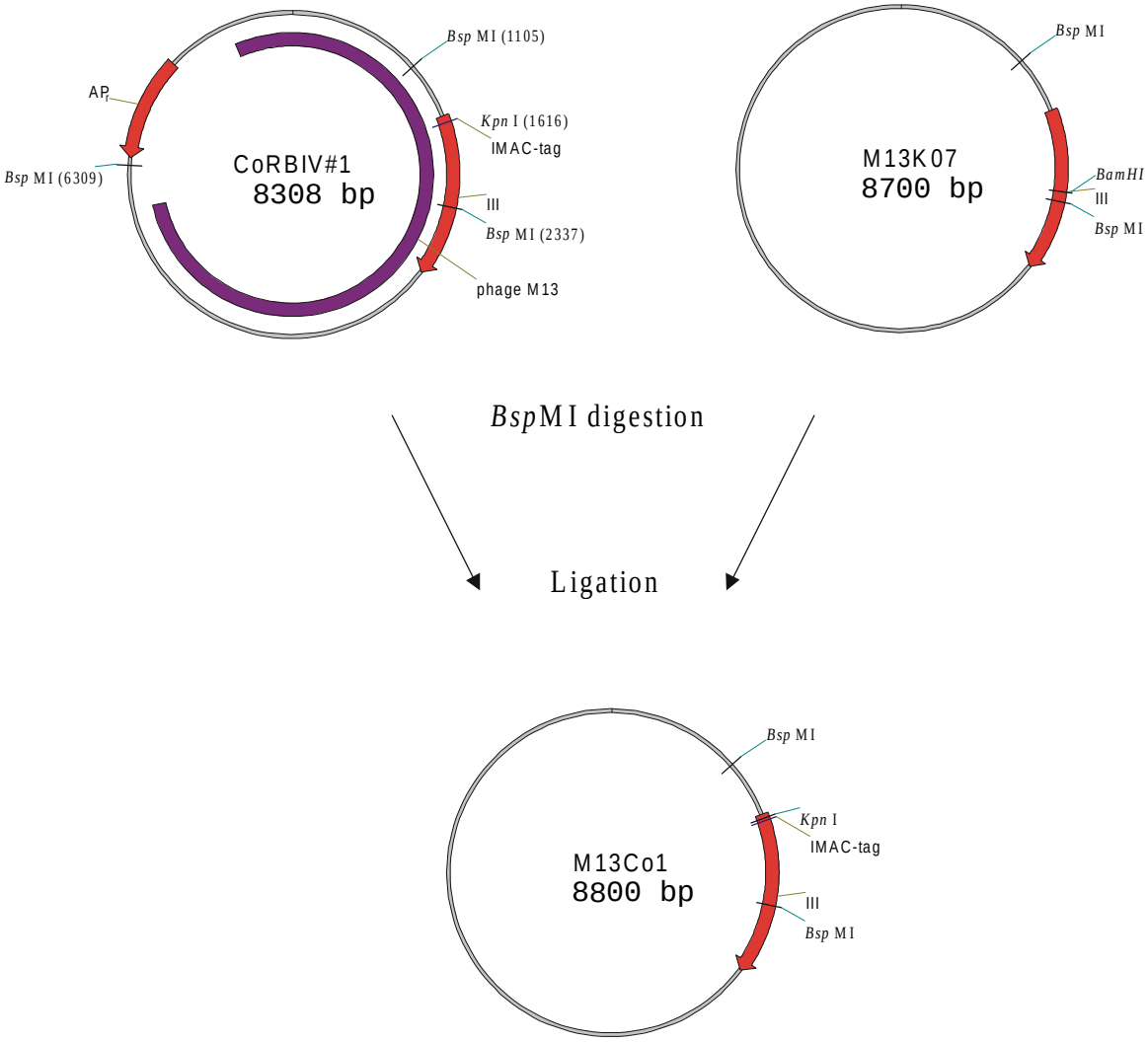


Figure 2.40: Cloning scheme of helper phage M13Co1

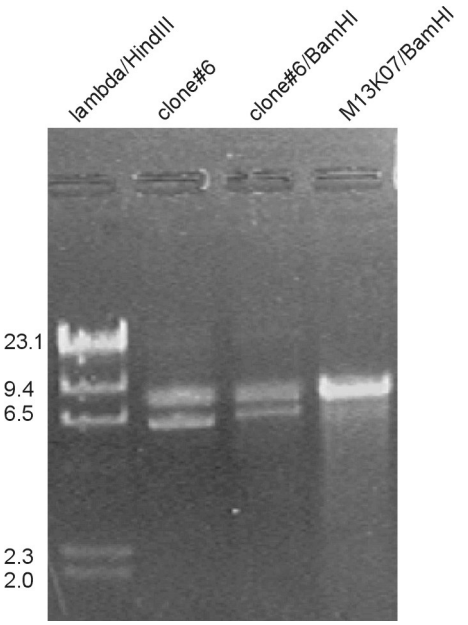


Figure 2.41: Restriction analysis of M13Co1

Helper phage analysis

Supernatant from the initial 3ml overnight culture from M13Co1 in XL-1 blue was transferred to a log culture of 20ml LB Sm50 of JM103 to allow infection. After incubation at 37°C, kanamycin was added to a concentration of 100µg/ml and the culture was incubated over night at 28°C on a shaker. Phage were prepared by PEG/NaCl precipitation. A panning was carried through on Ni(II) ReactiBind under standard conditions (5 x 400µl wash-PBS+imidazole). M13K07 served as a control.

Clone	Total input (cfu)	Total eluate (cfu)	Recovery (eluate/input)
M13Co1	$7x10^{8}$	$3x10^{5}$	$4.6x10^{-3}$
M13K07	$1.2x10^{7}$	<100	$<10^{-5}$

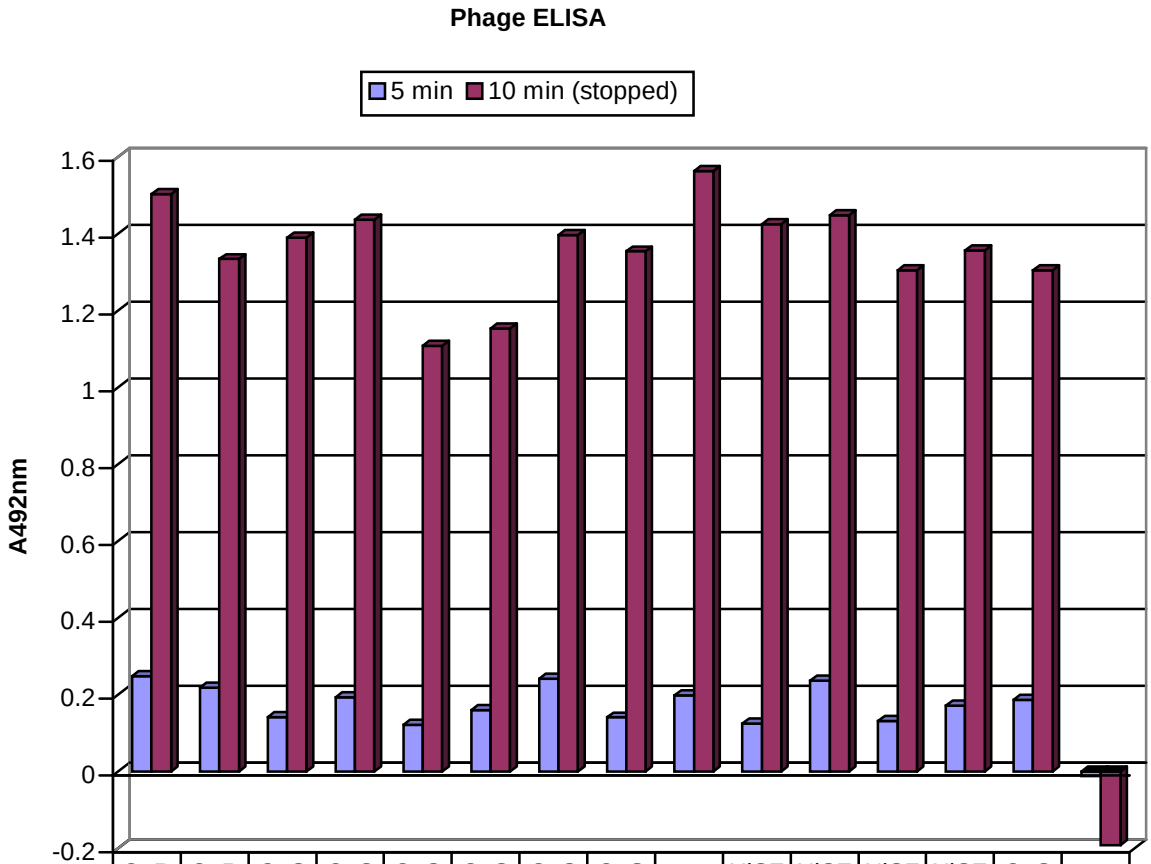
This result suggests a specific binding of the M13Co1 clone to Ni(II) if compared to M13K07.

2.7 Detection of metal ion binding variants

In order to save time, so-called phage-ELISA can substitute the tedious titre estimations if only the relative ranking of different phage variants in terms of binding is of interest. Therefore, several approaches for the detection of these variants were tested.

2.7.1 Detection by anti-M13 antibodies

Fourteen different variants of the transition metal binding phage were assayed on a nickel(II)-ReactiBind plate.



	CoR BIV# 1	CoR BIV# 3	CoS ZIV# 4	CoS ZIV# 5	CoS ZIV# 11	CuS ZIV# 11	CuS ZIV# 14	CuS ZIV# 15	NiRB IV#1	NiSZ IV#1 6	NiSZ IV#1 7	NiSZ IV#1 8	NiSZ IV#2 0	CoS ZIV# 7	M13 K07
5 min	0.25	0.22	0.14	0.19	0.12	0.16	0.24	0.14	0.2	0.13	0.24	0.13	0.17	0.19	-0.01
10 min (stopped)	1.5	1.33	1.39	1.44	1.11	1.15	1.4	1.35	1.56	1.43	1.45	1.31	1.36	1.31	-0.19

Figure 2.42: Phage ELISA of clones binding to Ni(II)-ReactiBind. Detection by anti-M13-HRP conjugate

As the affinities of the clones are exceptionally high, this detection method is not suitable to distinguish between the variants in terms of binding to the chelate.

2.7.2 Detection by a fluorescent chelate

As the author has invented a novel fluorescent chelating dye to detect phosphorylated peptides and proteins, it was interesting to show whether this compound may serve as a specific probe of metal binding peptides. The chelating dye comes readily complexed with iron(III) and was tested for affinity to FeSZIV#4. Opaque Maxisorp plates were coated with Fe(III) and Al(III) binding variants over night at 4°C. The Fe(III)-chelating dye was added to each well in 200µl MOPS .2M NaCl pH7.4 and incubated for one hour at room temperature. After two washes with MOPS .2M NaCl pH7.4 .05% Tween 20, the samples were subjected to fluorescence measurement. Two further measurements were made, one after two washing steps with the previous buffer, the other with this buffer containing 20mM imidazole.

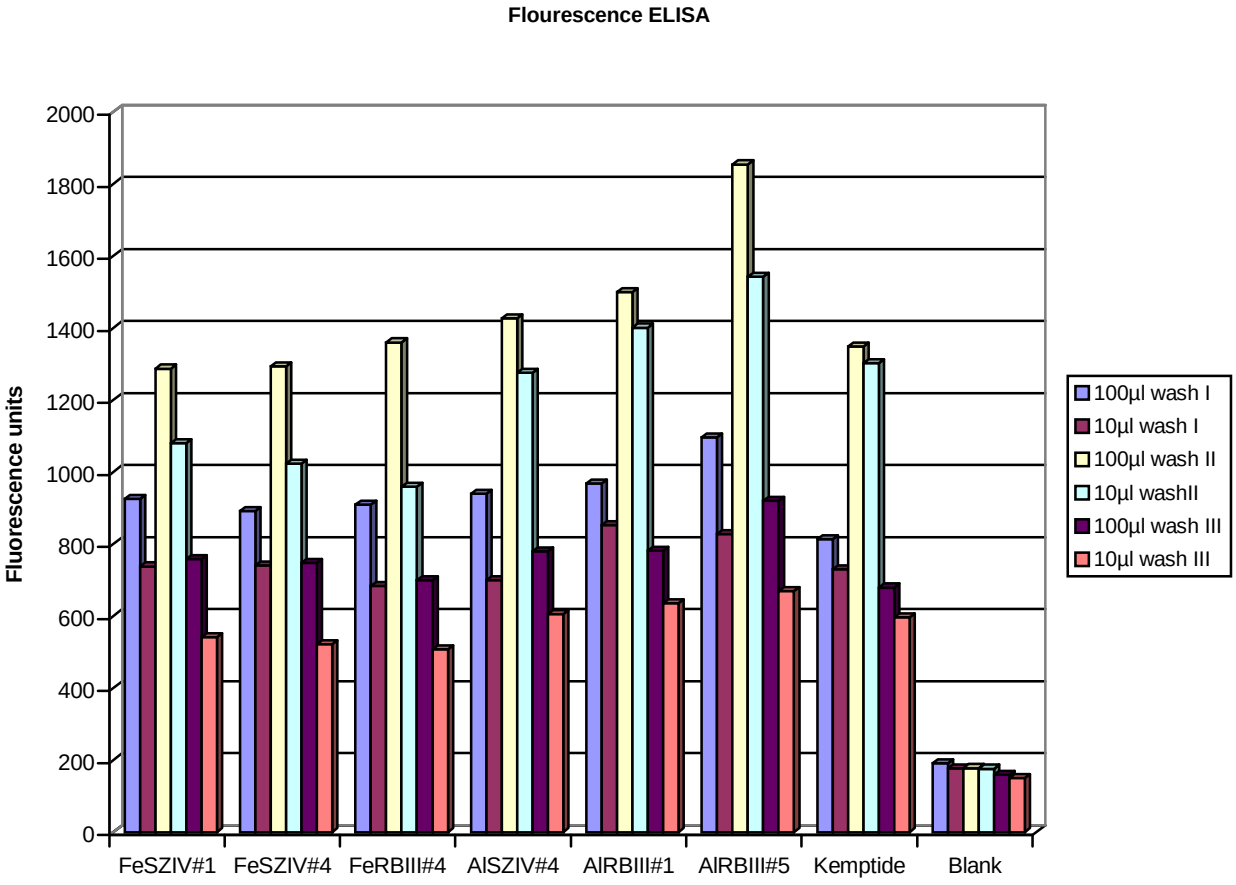


Fig 2.43: Fluorescence ELISA with different clones immobilised to Maxisorp microtitre plate. Detection by Fe(III)-IDA-FITC conjugate.

3 DISCUSSION

3.1 General selection strategy

Several conditions for successful protein or peptide purifications with IMAC are well described and were considered in terms of compatibility with phage display selection procedures. Buffers should be non-chelating and have high ionic strength to minimise background binding due to electrostatic interactions (Winzerling *et al.*, 1992). The most widely used affinity materials for the immobilisation of metal ions are IDA or NTA bound covalently to a resin-like support. Since cross-linked resins such as sepharose or superose have a molecular size exclusion limit, the capacity of this material is expected to be low in the context with filamentous phage as only the surface of the beads are accessible. Furthermore, comparing this material to supports like cellulose membranes or the smooth surface of polystyrene in microtitre wells, the void volume is very high. Therefore, a high background binding of non-specific phage variants is observed and extensive washing and further selection rounds are necessary (Barbas *et al.*, 1993). Thus, the commercially available affinity materials SpinZyme with a cellulose membrane modified with IDA groups and Reacti-Bind microtitre plates with chelating INDIA groups were chosen for the immobilisation of metal ions and affinity selection procedures.

Phage binding to the support of the affinity ligands causing an unspecific background can be avoided by pre-blocking with protein, like skimmed milk or BSA, and the application of non-ionic detergents such as Tween 20. In many standard phage display selection procedures, stringency imposed on the phage population in terms of binding to the affinity ligand is increased over the selection round. This diminishes the background of non-specific phage and favours the enrichment for the most avidly binding phage variants. Stringency can be applied by increasing the amount of washing steps, reducing the time allowed for the ligand interaction, reducing the amount of ligand presented or the introduction of a competitive ligand (Meulemans *et al.*, 1994; Levitan, 1998). At least the washing steps were increased during the selection rounds in all of the selection procedures. Elution of the phage population bound to the affinity ligand can be achieved by several strategies. The most common, but often least specific, is the elution by the application of an acidic buffer mainly at pH2 (Balass *et al.*, 1996). This is recommended if the mode of interaction is unknown, or other strategies are unavailable. However, filamentous phage are not stable in such an acidic environment for a long time and have to be neutralised after elution in order not to loose specific variants. The most favourable strategy is the elution with a competitive ligand which directs the phage population to the desired mode of interaction with the immobilised target. Another way

to elute the immobilised phage is the treatment with a protease cleaving an engineered site in the displayed protein (Cui *et al.*, 1996).

As the peptides identified by this affinity selection using IMAC were thought to be applied as affinity tags for protein purification, mild conditions for the whole panning process were chosen. Another prerequisite for a valuable affinity tag is the expression level and stability in the context with a recombinant fusion protein. In order to enhance the selection of stable and well-expressed peptides, a phage vector displaying only fused pIII coat proteins in contrast to a phagemid was chosen. Since infection of E. coli with filamentous phage is dependent on functional pIII fusions, the displayed peptide must not interfere with the folding of the protein and lead to a steric hindrance for the interaction with the host receptor proteins (Cesareni *et al.*, 1996). The cultivation of infected E. coli in liquid medium rather than amplifying the clones separately in colonies first on a petri dish, puts the population under a strong ecological pressure. This may lead to the enrichment of highly infective and good expressing phage variants.

3.2 Transition metal ion binding peptide variants

3.2.1 Affinity selection

It is known from IMAC experiments that the affinity of peptides and proteins to transition metal ions is best at a neutral pH range (Johnson et al., 1996). Sodium chloride suppresses electrostatic interactions and reduced background binding. Often, phosphate containing buffers like PBS are recommended. Therefore, PBS containing .5M NaCl and .05% Tween 20 was the buffer of choice. Blocking was performed by the addition of 3% BSA before and during the incubation with the phage and also for the 1st washing step. As the histidine side chain incorporates imidazole which interacts strongly with the transition metal ion, .5M imidazole was used as a competitor for the elution of specifically retained phage variants. In order to increase the stringency on the phage populations in the consecutive rounds, 20mM imidazole was added to the washing buffer in rounds II-IV. Similarly, the amount of washing steps were increased in rounds III-IV from 5 to 10 for SpinZyme and 3 to 5 for ReactiBind. Initially, more than 20 clones were sequenced from the 4th round of the first pannings (Tables 2.1 and 2.2). It became obvious that the diversity is very limited after the selection rounds. Therefore, 5 to 15 randomly picked clones were found to be sufficient for the characterisation of the enriched population, especially for ReactiBind selections (Tables 2.9-2.13).

The comparison of recoveries from round I and IV in all of the performed selections showed a clear increase. The comparison of the recovery rates of the 4th rounds displayed a ranking of metal ions comparable to the literature known for IDA chelates. In the case of cobalt(II)-SpinZyme, it was shown that the recovery rates are clearly dependent on the imidazole concentration present in the washing buffer (Figure 2.2). The analysis of sequences found in the isolated clones from round IV of all transition metal ion selections all contained a multitude of histidine residues ranging from 4 to 6 in the random 15-mer. For the random sequences is encoded as NNS, only one codon (CAC) out of 32 possible can encode histidine. The diversity of the peptide bank is reported to be in the range of $2x10^7$ individual clones (Devlin *et al.*, 1990). The chance of 6 histidine codons occurring in one clone is thus one in $1.07x10^9$. This exceeds the diversity by a factor of about 50 which must be attributed either to an asymmetry in the codon distribution or to mutations accumulating during the panning procedure. The latter can be almost excluded since several identical clones have been obtained from separate selections. This also indicates that the clones selected were unique in the library containing as many as 6 histidine codons. Therefore, a significant factor for the interaction of the selected peptides can be considered to reside within the histidine residues. From previous experiments it is known that amino acids like histidine, cysteine, tryptophan and arginine bind to Ni(II)-IDA strongly in a decreasing order (Hemdan and Porath, 1985). Cysteine residues were not found in any of the selected sequences, probably due to the aggregation of pIII caused by unpaired cysteine forming covalent bonds with other proteins in the periplasm of E. coli. Tryptophan, being encoded only by 4 of 16 different clones, thus may not play an important role for the binding. Arginine residues occur more often and are embedded in histidine clusters. The histidine rich sequences can be separated into continuous and discontinuous sequences. Five clones display a stretch of 4 consecutive histidines.

Table 3.1: Alignment of continuous histidine motif. Histidine is printed in bold.

CoSZIV#4	ALPRSSP**HHHH**LP**H**R
CoSZIV#5	MGSN**H**M**HHHH**FP**H**LP
NiSZIV#16	LD**H**TYRA**H**SKV**HHHH**
NiSZIV#18	Y**H**TSI**HHHH**PVD**H**LA
NiRBIV#1	**HHHH**SYMSSIPSTAW

The stretches are flanked by proline and hydrophobic amino acids. The other selected sequences do not seem to display such a specific motif. Studies have been made

concerning the positioning of histidine residues in a peptide in its ability to bind metal ions. The adopted structure of such a sequence determines the spacing necessary for binding (Arnold and Haymore, 1991; Haymore *et al.*, 1992). In principle, α-helices chelating metal ions have a spacing of two amino acids between the histidines. The other structures β-fold and β-turn are not as likely to be adopted in such a short peptide. Looking at the selected sequences with gapped histidines, all kinds of spacings can be found, suggesting that the displayed peptides are mainly disordered in terms of structure. Basically, this distribution of a prominent amino acid resembles the peptides selected as plastic binders (Adey et al., 1995; Gebhardt et al., 1996) which incorporate tryptophan, tyrosine and arginine residues.

3.2.2 Properties

Naturally occurring transition metal binding peptides and proteins have a specificity for their ligand. A zinc finger domain, for example, coordinates the zinc(II) by two histidines and two cysteine groups. The dissociation constant of such an 18mer peptide for zinc is about five orders of magnitude lower than for cobalt (Bavoso et al., 1998). Therefore, strong preference for zinc(II) can be observed.

Some of the metal ion binding clones were selected from panning on other transition metal ions (Figure 2.27), suggesting that specificity of the sequences for a metal ion and the immobilising support may be low in general. Therefore, one clone selected from each of the four metal ions and the two support materials were checked for their relative binding to any of the ligands (Figures 2.28 and 2.29). As a result, mainly those clones which were selected on the specific affinity material exhibited the highest recovery rates on those. Best recovery rates were found with clones containing a continuous histidine motif. Interestingly, N-terminal his-motif prefers clearly Ni(II)-INDIA, whereas an internal his-motif favours Cu(II)-IDA chelates. Discontinuous motifs bound weaker to the affinity material, but were biased towards the chelating groups they were previously selected on. Iron is a very poor ligand for the peptides and shows some binding in the context with ReactiBind. This clearly demonstrates the strong influence of the chelating group. Note that the panning on Fe(III) was performed in absence of imidazole. It can be expected that the residual binding of clones can be quenched by the introduction of the competitor.

3.2.2.1 Clones selected from ReactiBind

NiRBIV#1, the only clone with continuous histidine motif, bound to all ReactiBind metals but zinc(II) with the highest affinity of all. Especially, to cobalt(II) and nickel(II) on which the clone was selected. Furthermore, this clone did not display a good binding

to the metal ions immobilised with SpinZyme, but at least with the preference for nickel(II). This demonstrates the selectivity for the immobilising chelate as well as for the bound metal ion.

CoRBIV#3 and CoRBIV#1 were best retained to nickel(II) on ReactiBind, but were a poor ligand for the other metal ions, even though they were selected on Co(II)-RB and Co(II)+Cu(II)-RB, respectively. The difference to the other clones were more marked on SpinZyme, where recoveries of CoRBIV#3 were 2 or even more orders of magnitude lower than for the strongest binder. Both of these clones share a discontinuous histidine motif with a maximum of two grouped histidines.

CoSZIV#11 was the most promiscuous clone which was selected from all affinity ligands but zinc(II). This was reflected by an average binding to ReactiBind and SpinZyme. The only exception was Zn(II)-RB where this clone was the best binder, which cannot be explained sufficiently from the selection. The recoveries observed during the selection rounds on Zn(II)-RB were very unstable and may be due to a loss of a prominent clone, which could be accounted to CoSZIV#11.

Table 3.2: Properties of clones binding to transition metals on INDIA

Specificity class	Sequence	Ranking	Charge	His
Ni(II) > Zn(II) > Co(II) > Cu(II)	**HHHH**SYMSSIPSTAW	1	0	4
	MGSN**H**M**HHHH**FP**H**LP	2	0	6
Zn(II) > Ni(II) > Co(II) > Cu(II)	**H**R**HH**RP**H**GDT**H**RVTP	3	+2	5
Ni(II) > Cu(II) > Zn(II) > Co(II)	A**H**QQT**HH**YFT**HH**LNY	4	1	5
Ni(II) > Zn(II) > Cu(II) > Co(II)	VA**HH**WW**H**DGYK**H**PLN	5	0	4
	A**H**P**H**R**HH**SDSMLVT**H**	6	0	5
	APS**HH**T**H**S**HH**LTQMR	8	1	5
Ni(II) > Co(II) > Zn(II) > Cu(II)	ALPRSSP**HHHH**LP**H**R	7	+2	5

From this ranking by affinity and specificity, some properties for binding can be deduced. Continuous stretches of histidine residues at the N-terminus without alanine and two consecutive hydrophobic amino acids are favoured by INDIA bound metals. Dispersed histidines not joined by amino acids with either hydrophobic or amino group containing side chain are less retained by the affinity material. All of these clones bound

to iron(III) only poorly, unless it was immobilised on ReactiBind, suggesting a high background binding to this affinity material alone, or altered properties of iron(III) immobilised by INDIA chelate.

Fast Lane

As panning on transition metals led to phage variants with very strong affinities, a shorter protocol was tested by omitting the PEG/NaCl precipitation of phage particles. The phage were applied directly from the supernatant of the overnight cultures. This was thought to save time and approach the more complex conditions relevant for the protein purifications from E. coli, thus yielding appropriate affinity peptides. No considerable enrichment over the selection rounds was found, though nickel(II) served as a ligand. It is most likely that citrate or other dicarbonic acids present in an overnight culture scavenged the metal ion from the support. Generally, a tetradentate chelating group like NTA should form a more stable complex under such conditions.

3.2.2.2 Clones selected from SpinZyme

CoSZIV#5 contains a continuous histidine motif. Though it was not selected on nickel(II), it is the best binder for all metal ions immobilised on SpinZyme with strongest preference for copper(II). Recoveries from metal ions on ReactiBind were mainly lower than those of other clones selected on this material with best results from Ni-RB.

CoSZIV#4 has a continuous motif similar to CoSZIV#5 and displays a comparable preferences for the affinity ligands, too. The efficiency of binding is only about threefold lower.

CuSZIV#11, clone which was identified only once on Cu(II)-SZ selection, displayed poor recovery rates on ReactiBind except for Ni(II)-RB. The recoveries on Ni(II) and Cu(II)-SZ were more elevated.

NiSZIV#17, a minor clone from the Ni(II)-SZ selection, was the worst binder all other tested clones on ReactiBind. Best interactions were discovered for Ni(II)-SZ, about $1/5^{th}$ of CoSZIV#5. The other metal ions led to recoveries in the range of the ReactiBind clones. Therefore, this clone displayed a very high specificity for its own selection material.

Table 3.3: Properties of clones binding to transition metals on IDA

Specificity class	Sequence	Ranking	Charge	His
Cu(II) > Ni(II) > Co(II) > Zn(II)	MGSN**H**M**HHHH**FP**H**LP	1	0	6
Ni(II) > Cu(II) > Co(II) > Zn(II)	ALPRSSP**HHHH**LP**H**R	2	+2	5
	A**H**P**H**R**HH**SDSMLVT**H**	3	0	5
	HR**HH**RP**H**GDT**H**RVTP	4	+2	5
	APS**HH**T**H**S**HH**LTQMR	5	1	5
	A**H**QQT**HH**YFT**HH**LNY	6	0	5
Ni(II) > Co(II) > Zn(II) > Cu(II)	**HHHH**SYMSSIPSTAW	7	0	4
Ni(II) > Cu(II) > Zn(II) > Co(II)	VA**HH**WW**H**DGYK**H**PLN	8	0	4

Positioning and number of histidines, placement of few hydrophobic groups are very important characteristics for transition metal binding peptides. These properties determine the avidity of binding and the preference for certain metal ions. The best clone for IDA bound metals possesses a central continuous stretch of histidine residues flanked by hydrophobic groups. It is the only clone which prefers copper(II) over nickel(II). The other clones with stronger binding to nickel(II) possess an alanine at the N-terminus, suggesting that the α-amino-group participates in the binding process (Sulkowski, 1985). All recoveries on Fe(III)-SZ were about 3 to 4 orders of magnitude lower compared to Ni(II)-SZ.

3.3 Hard Lewis acid binding peptide variants

Hard Lewis acids are often immobilised in enzymes by chelating organic compounds such as porphyrines, carbonic acid side chains, alcohol groups or even the oxygen from the peptidic backbone. Fe(III) is additionally bound by cysteine groups acting as clusters, too. Purification experiments have shown a bias of Fe(III) and Al(III)-IDA for primary phosphate groups as preferred ligands under acidic conditions (Muszynska *et al.*, 1986; Andersson, 1991). However, this preference is strongly dependent on pH value and phospate content of the buffer (Muszynska *et al.*, 1986). Comparable to IMAC with transition metal ions, high salt concentrations can reduce the non-specific

electrostatic interactions (Zachariou, 1996). In order to find novel specific affinity handles for immobilised hard Lewis acids, conditions were chosen under which no binding to phosphate groups (MOPS-buffer at pH7.4) and only mild electrostatic interactions (.2M NaCl) were expected. In order to reduce the background binding of phage to the material, blocking was carried through by 3%BSA and the addition of .05% Tween 20 in all buffers. BSA itself is known to bind to Fe(III)-IDA under acidic conditions (Sulkowski, 1988). Nevertheless, it could act as an additional factor for stringency when panning was carried through at pH7.4. As for the successful affinity selection on transition metals, the same amount of washing steps, volumes and selection rounds were applied. Since the mode of binding of peptides to the hard Lewis acids was unknown, 50mM EDTA served as an elution buffer, removing both the metal ion and the phage from the support material. Titanium was an exception, because it is very reactive with water and it was not sure whether it was properly immobilised on the chelating groups or was even present as an oxide. Therefore, the classical approach for elution, the acidic glycine buffer pH2.2 was chosen. All of the hard Lewis acids were chosen with respect to their low toxicity for their possible future application as affinity handles for the purification of pharmaceutically relevant peptides or proteins. Especially titanium is interesting due to its multiple applications for prostheses. If a peptide could be found binding to this metal or its oxides, a better integration of this material into existing tissue could be achieved.

3.3.1 Affinity selection

Most of the affinity selections were carried through with SpinZyme as the chelating support. Selection on Al(III) and Ce(IV) were initially performed in presence of PBS because no phosphate containing peptides should be selected. The recovery of Ce(IV)-SZ was very high in the 1st selection round ($2.6x10^{-4}$), suggesting a very high background binding of phage to the immobilised metal ion. The recovery rates in the consecutive cycles remained constant, and probably all peptide-displaying phage were lost during the selection since no selective advantage of a specific peptide-variant could compete for the wild-type phage. Al(III)-SZ displayed a weaker background binding, but also showed no considerable enrichment during the selection rounds. Therefore, MOPS was used to replace for PBS which could probably interfere with the interaction of peptides with the metal ion. The recovery rates even deteriorated over the selection rounds with this buffer. Similar results were obtained by affinity selection on magnesium(II) and calcium(II). Sequencing revealed many deletions with both MgSZIV and AlSZIV only containing 1 insert out of 5 clones. Fe(III)-SZ selection resulted in an increase of recovery rates by about 50-fold which then decreased in the 4th round, suggesting a strong interaction with the affinity material. Sequencing of Fe(III)-

SZ selected led to the discovery of peptides with 2 to 4 histidine and 1 to 3 lysine residues. Lysine, like histidine has only one codon allowed in an NNS random sequence (AAG). Therefore, the sequences are as likely to be found in the entire peptide bank as those selected on transition metal ions. Additional features are arginine, threonine and serine residues which could represent a novel binding motif for Fe(III)-IDA.

Table 3.2: Alignment of iron(III)-IDA binding peptides. Histidine is printed in bold, positive charges are underlined.

FeSZIV#4	GIPA**H**EQ**H**TKKLWLL
FeSZIV#1	WPTKKFTLT**H**K**H**SKR
FeSZIV#7	A**H**PS**HH**RAPSR**H**KSI
FeSZIV#14	LQSFGKLPYSRLYSV

Selection on ReactiBind resulted in intermediate increases in the 2nd and 4th round of selection with Ti(IV), Al(III) and Fe(III). Sequencing revealed peptides containing 3 to 4 histidine and 1 to 3 arginine residues. However, the positioning of these residues are too diverse to allow an alignment. The clone identified from the Ti(IV) selection was identical to CoSZIV#7. Imidazole did not alter the recovery on Ti(IV)-RB, suggesting that other residues of the peptide are more involved in the interaction.

3.3.2 Properties

In order to evaluate some of the promising sequences for their affinity to different ligands, a panning experiments for cross-reactivity were carried through. In Figure 2.30 the recoveries are displayed for direct comparison. Clone FeSZIV#1, due to its superior binding properties, was evaluated more extensively.

3.3.2.1 Cross-reactivity of hard Lewis acid binding variants

As shown for the clones identified from transition metal ion selections, best recoveries were found with the original selection material (ReactiBind or SpinZyme and the metal ion). FeSZIV#1 did bind exceptionally well to nickel(II) and iron(III), primarily when chelated with IDA with a recovery up to 23%. FeRBIV#1 was the second best binder on Fe(III)-RB and SZ with twice the affinity to ReactiBind over SpinZyme. It also showed considerable binding to Ni(II)-RB. Aluminium(III) seems to represent a weak ligand for interaction. Probably only a small fraction of ions are presented in a complex forming

state on the chelate at a neutral pH, especially because a 10mM $AlCl_3$ solution was observed to precipitate when adjusted to pH 7. Iron(III) shares similar features, but is more stable for a short period of time.

Table 3.6: Properties of clones binding to hard Lewis acids on IDA

Specificity class	Sequence	Ranking	Charge	His
Ni(II) > Fe(III) > Al(III)	WPTKKFTLT**H**K**H**SKR	1	5	2
Ni(II) > Fe(III) > Al(III)	QLPATT**H**FRAPLG	2	1	1
	GIPA**H**EQ**H**TKKLWLL	3	1	2
	QALFSSNFSFRGRLA	4	2	0
Fe(III) > Al(III) > Ni(II)	PPQKQ**H**ATFWP**H**F**H**N	5	1	3

General features determining the ranking seem to include the net charge, hydrophobicity and amount of histidines present in a peptide. A low hydrophobicity accompanied with a charge of +5, 2 histidines and 5 hydroxyl groups seem to favour a strong binding to nickel(II) and iron(III). Decreasing values of all these properties lead to a weaker binding.

Table 3.7: Properties of clones binding to hard Lewis acids on INDIA

Specificity class	Sequence	Ranking	Charge	His
Fe(III) > Ni(II) > Al(III)	WPTKKFTLT**H**K**H**SKR	1	5	2
	QLPATT**H**FRAPLG	2	1	1
	GIPA**H**EQ**H**TKKLWLL	5	1	2
Fe(III) > Al(III) > Ni(II)	PPQKQ**H**ATFWP**H**F**H**N	3	1	3
Ni(II) > Fe(III) > Al(III)	QALFSSNFSFRGRLA	4	2	0

Iron(III)-bound INDIA chelating groups are favoured by the same peptides which are more strongly retained on nickel(II) on IDA. This demonstrates the influence of the chelating support on the binding properties of metal ions (Winzerling et al., 1992).

3.3.2.2 FeSZIV#1 binding properties

As determined from the cross-reactivity tests in comparison with the other transition metal ions, FeSZIV#1 has unique binding properties for nickel(II) and iron(III). The recoveries of this clone in comparison to the next best clone FeRB#1, are 25-fold and 5 five orders of magnitude higher for Fe(III)-SZ and Ni(II)-SZ, respectively. In order to evaluate the interactions involved in the strong binding parameters like pH value, salt content blocking agents, buffers and competing chemicals were tested.

Lysine is known to bind Fe(III) (Schneider-Mergener *et al.*, 1996). Proteins like cytochrome c, containing many lysine residues, are usually desorbed from Fe(III)-IDA by the addition of only .3M NaCl (Zachariou and Hearn, 1996). This suggests that the affinity is based on electrostatic interaction with the hydrolytic complex of Fe(III). However, washing with .5M NaCl in MOPS did only reduce the recovery of the phage to 1/3rd compared to .2M. Competition with .1M lysine and .1M ethanolamine did decrease the recovery to 1/4th and a half, respectively. This shows that lysine is indeed involved in the interaction with iron(III). 20mM Tris buffer with .5M yields the same results as .5M NaCl MOPS pH7.4, indicating that low amounts of chelating groups do not affect the binding. A pH value of 8, however decreases the phage recovery by 1/5th. Therefore, lysine possessing a pKa of 10 and also the histidine residues (pKa=6) become deprotonated reducing the affinity.

Phosphate is a good complexing ligand for iron(III), especially at a low pH (Muszynska, et al., 1992). Elution can be performed by the use of a phosphate buffer. If the binding of the FeSZIV#1 clone were dependent on the phosphorylation of its His residues, PBS should decrease the recovery rate by far. Indeed, the yield of phage is only 13% of the same buffer with MOPS. The elevation of the salt concentration to 1M NaCl increases the yield almost 2-fold. Inhibitory sequences for a histidine-kinase, enzyme I of the phosphoenolpyruvate-sugar phosphotransferase system, were identified recently by phage display (Mukhija and Erni, 1997). These peptides contain histidine with an adjacent arginine are rich in basic residues and lack acidic amino acids. Another possibility for the decreasing recovery of phage by PBS could be that phosphate blocks other types of interaction with Fe(III) as well.

The blocking of the SpinZyme separation units with 2%skimmed milk powder in T-PBS .5M NaCl was not successful, because the membrane was plugged with protein particles and did not allow access to the matrix. No blocking and low ionic strength conditions resulted in a low recovery of about .5% of total input. This could be attributed to the presence of other proteins from the phage preparation competing with the affinity groups.

The application of 20mM imidazole to the washing buffer did affect the affinity to Fe(III)-IDA only marginally by a $1/6^{th}$ lower recovery. Therefore, the histidine residues are not as responsible as the lysines.

Another experiment showed an even stronger binding to Ni(II)-IDA than to Fe(III)-IDA, indicating that Fe(III) may share some complexing features for histidine with nickel(II). However, addition of 20mM imidazole to the washing buffer abolished all binding of the phage to the transition metals Ni(II), Cu(II), Co(II) and Zn(II) immobilised on IDA below the detectable limit.

The properties of the FeSZIV#1 clone suggests a cooperative contribution of several amino acids to the binding to Fe(III)-IDA. The main binding strength seems to be provided by the positively charged amino acids lysine and arginine. The histidines also participate in the interaction, as can be seen by the strong binding to Ni(II)-IDA. Such a binding is only to be expected from a selection process, since the 2 His residues have to be placed properly for binding. Indeed, phage display with a random hexamer peptide bank yielded mainly peptides with two histidines when selected in absence of imidazole (Patwardhan *et al*., 1997).

3.4 Applications for metal affinity peptides

As the hexahistidine-tag was found to bind strongly to transition metal chelates, a multitude of applications have been devised exploiting the binding properties. In order to demonstrate a comparable usefulness for the new identified metal ion binding sequences, experiments including a model protein purification under denaturing conditions, phage IMAC purification, construction of an IMAC helper phage and detection of peptide fusion were made.

3.4.1 Protein purification

One of the major advantages of a hexahistidine tag compared to other affinity handles such as glutathione-S-transferase (GST) fusions , is the stability of the interaction under denaturing conditions (Volkel *et al*., 1998). This allows both the purification of proteins from aggregates such as inclusion bodies as well as refolding of denatured proteins directly on an affinity clolumn. As the phage variants selected by IMAC display a fusion pIII-protein on the surface, disruption of the particles was attempted by the addition of different urea concentrations. Subsequent purification was performed using several metal chelating materials. Only the tetradentate materials Ni(II)-NTA and Co(II)-Talon were found to be compatible with high urea concentrations (2-8M). Ni(II)-NTA yielded the best recoveries and was therefore used for the further experiments.

CoSZIV#7 was assayed for the binding under denaturing conditions in order to evaluate the requirement of structure for binding to the metal ion. The efficiency was best with 2M urea concentration and decreased by one half at 6M (Figure 2.32). This demonstrates the absence or at least low influence of a structure for the interaction. As expected, some of the pIII protein did remain bound to the resin due to its hydrophobic C-terminus and could be eluted with 1% SDS.

Optimisation was attempted by using different washing conditions and monitoring the results by both western blot and silver-stain. The application of 20mM imidazole during the wash did result in less pVIII contamination in the eluted samples (Figure 2.33a) which was even better with 4ml wash compared to 1ml. The input sample S represents the amount of phage protein visible if it were completely retained and eluted from the resin. It becomes clearly visible that only traces of the pVIII protein are present in the elution, more than 2 orders of magnitude less than from the total input. As pVIII is also hydrophobic, residual amounts are removed with 1%SDS. The parallel western blot (Figure 2.33b) developed by anti-pIII-antibody shows similar amounts of pIII-fusion in all the eluted fraction, with a little less presence in the 1%SDS fractions. The amount of pIII-fusion protein recovered by the affinity purification may represent 5-10% of the total input. As other background proteins disappear after the purification, an enrichment of the fusion protein can be observed.

In order to evaluate the application of the novel iron(III) binding peptide FeSZIV#1 as an alternative for the conventional hexahistidine tag, a purification of the pIII fusion protein like in the previous chapter with CoSZIV#7 was attempted. Though it was not clear whether an other iron(III) chelate other than IDA would be applicable, NTA was chosen for the immobilisation because of its stability in the presence of high molarities of urea. It is important to charge the resin with $FeCl_3$ shortly before the chromatography since the ion forms oxides after prolonged exposure at a neutral pH. The results obtained are very comparable to the previous purification of CoSZIV#7. The silver stain does not show any traces of pVIII or other contaminating proteins which can be seen in the input sample S (Figure 2.34). Only a faint band in the range of pIII is visible. The western blot developed with both a pIII and pVIII (anti-M13) antibody, demonstrates the recovery of about 10% pIII protein at any urea concentration (Figure 2.35). The pVIII protein is undetectable in the elution samples. However, the anti-M13 antibody is reactive against native pVIII coat protein and is therefore only weakly sensitive to the denatured protein on nitrocellulose. The sensitivity of this antibody can be compared with a coomassie stain. Nevertheless, enrichment of the fusion protein can be demonstrated and will probably become useful as a novel affinity handle in the future.

Another purification was performed for direct comparison of both affinity tags CoSZIV#5 which is the best ligand for transition metals on IDA and FeSZIV#1 being the best binder for the hard Lewis acids on IDA. This time, the amount of phage applied was greater in order to evaluate the co-purification of background proteins. The result was a strong reduction of the background in both samples by at least a factor of 100 which can be seen on the coomassie stain (Figure 2.36). The recovery of pIII protein is good (~15%) in both of the purifications. Still, the backgound needs to be reduced, which shall be feasible by introducing more washing steps than only 1ml 8M urea. It is not clear why the pIII protein was eluted in the 1%SDS step only. Probably the void volume of the manually prepared column was different prom the previous purifications. It could be demonstrated that the affinity tags identified by phage display were functional under conditions comparable to conventional protein purification under denaturing conditions. Thus, it can be deduced that a structure for the affinity peptide is not necessary for the interaction and should be useful tools for the purification of recombinant proteins.

3.4.2 Helper-phage

For the technique of phage-display, sometimes pure phage preparations free of residual E. coli proteins are necessary. Panning on whole cells has the necessity of bacteria and toxin free phage populations which do not interfere with the metabolism of eucaryotic cells, creating artefacts. The classical way to remove these contaminations from a phage preparation is a gradient centrifugation by which the phage are concentrated in a discrete band in CsCl. The disadvantage is the time needed and a relative low yield with such a separation method. Another way of reducing contaminants is a repeated PEG/NaCl precipitation and resuspension which also results in a loss of phage and will eventually concentrate co-precipitated proteins. When it became obvious from the panning results that high recoveries can be obtained by an IMAC with metal ion binding phage, several affinity materials and protocols were tested for the use of phage preparation.

The objective was an affinity purification directly from the supernatant of the bacterial culture. As discovered before in other experiments (Fast Lane), tridentate supports were not stable enough to retain the metal ions in complex mixtures such as bacterila culture supernatants. The Chelating Sepharose FF did display a recovery of $5x10^{-4}$ at the best, which causes a great loss of phage. The best result was obtained with Talon, retaining about .7% of the input phage. This demonstrates the superior stability of the tetradentate chelates, but resins are not the best material for the phage preparation. The reason for this may be the size exclusion of the resin material, which allows only the surface association of phage, reducing the total capacity immensely. The material of choice

should be the SpinZyme affinity separation unit, by which phage can be recovered to more than 20% with nickel(II) and 10% with iron(III). Leeching of the metal ion from the support can be avoided by still performing a classical PEG/NaCl precipitation first. Therefore, a helper phage was constructed to be used in the future when a quick and simple phage preparation needs to be performed. Since the M13LP67 vector and M13K07 helper phage are both derivatives of the M13 phage, some of the restriction sites around the pIII gene are identical. The type IIs restriction enzyme was chosen to easily sub-clone the affinity tag sequence of CoRBIV#1 unambiguously into M13K07 (Figure 2.40). This resulted in a phage which displayed the correct restriction sites (Figure 2.41) and molecular weight conferring the kanamycin resistance gene. This helper phage, designated M13Co1, was shown to be retained on Ni(II)-RB to a similar extent as the parent phage under standard conditions (.5%). M13K07 was assayed in parallel, but was not detectable in the eluate and has therefore a recovery rate below 10^5. This procedure of cloning facilitates the grafting of pIII displayed peptides from M13LP67 to M13K07 in order to prepare phagemid particles with altered binding properties.

3.4.3 Detection

Many recombinant fusion proteins have the advantage to be detected by standardised methods due to a specific ligand directed against the fusion peptide/domain. One of the most established techniques for quantification is the ELISA. In phage display, the avidity of binding to the ligand is evaluated via a so-called phage-ELISA. The ligand is immobilised to the surface of a microtitre plate in serial dilutions which is blocked against unspecific interactions. For the evaluation of the enriched populations and single clones, a quick and simple method was to be devised. Therefore, a phage-ELISA using Reacti-Bind microtitre plates charged with nickel(II) was performed. However, the phage were bound tightly to the chelate an did result in very strong signals after only 5 to 10 min of incubation with the ELISA-stain. The differences in the signal were to low to discriminate the efficiency of each single clone in terms of binding to the metal. Furthermore, it became clear that not only the metal ion, but also the immobilising chelating group influence the avidity of the displayed peptide. Thus, every interaction of single clones with each affinity material was evaluated by the titre estimation of input and output.

A new compound was invented and synthesised during the course of the PhD thesis. The Fe(III)-IDA-FITC conjugate was shown to label phosphorylated peptides on beads specifically (to be published). This specific interaction can be detected by fluorescence emission of the fluoresceine moiety. As especially FeSZIV#1 was found to bind to Fe(III)-IDA tightly, this clone and other hard Lewis acid binding variants were assayed

for the interaction with the chelating dye. Unfortunately, the background was considerably high and was not reduced sufficiently after several washing steps. The problem may result from the femtomolar concentration of the displayed peptides which is at the detection threshold of fluoresceine.

4 PROSPECTS

The affinity peptides for immobilised metal ions identified have been characterised in the context with the phage particles so far. Several parameters remain to be characterised. As the phage presents 5 pIII-fusion peptides on each particle (polyvalent), a phage-ELISA cannot be used to determine the dissociation constant. Therefore, either a new fusion protein will have to be devised, or synthetic peptides which can be assayed by a competition ELISA or surface plasmon resonance. It will be interesting to evaluate the dissociation constants in comparison with the hexahistidine sequence for which confusing values are given, ranging from 10^{-6} to 10^{-13} M (Loetscher *et al.*, 1992; Nieba *et al.*, 1997; Kommissarov *et al.*, 1996).

A fusion protein with a reporter enzyme such as peroxidase could also be applied for test purifications to optimise the protocols established so far. Furthermore, it is important to evaluate the expression level, because histidine belongs to the rare amino acids and may cause the translation apparatus to stall and result in misincorporation if the supply of aminoacylated tRNA becomes short (Ulrich *et al.*, 1991). The compatibility with the folding of protein domains needs to be tested.

The effectivity of translocation of such a fusion protein is important for secretory proteins. The highly positive charged iron(III) tags resemble sequences next to a stop transfer sequence which causes secreted proteins to remain intergrated in the membrane and determine the topology (Anderson et al., 1992). Interestingly, no N-terminal, only C-terminal secretable hexahistidine-fusion proteins in E. coli were found in the literature, suggesting interference with the translocation process. As the pIII protein is Sec dependent, it is interesting how this problem is solved in the morphogenesis of the phage particle (Marvin, 1998). This also leaves the C-terminal orientation of a fusion protein to be tested for only N-terminal fusion with a polyproline-linker to pIII was present in the selection processes.

Higher amounts of fusion protein can be assayed for specific detection via the chelating dye which will not necessarily be complexed with Fe(III), but with any of the other metal ions. Many phage display vectors possess a fusion of the displayed protein, a histidine-tag with an amber stop and pIII coat protein. The production of soluble protein is performed in a non-suppressing strain. The subsequent purification is achieved with IMAC of the histidine-tag fusion. Often further evaluation of the binding properties of such phage involve FACS analysis. A major problem is the labelling of these small molecules with fluoresceine, because covalent modification of lysine residues often

leads to aberrant folding or masking of the binding surface. This problem is usually circumvented by a secondary labelling with a fluoresceine-stained antibody directed against a tag fused to the sc-Fv. The chelating dye will only bind to the purification tag and will therefore not be involved in the interaction of interest. The stoichiometry is also clear, because only one molecule can bind to one tag sequence. As the cross-reactivity between the nickel(II) and iron(III) is very low, simultaneous detection of differentially tagged proteins with chelating dyes can be performed.

Procedures such as crystallisation for the structure determination of proteins require homogeneous preparations. The hexahistidine-tag can be used to purify the recombinant fusion protein to near homogeneity. A second affinity tag is then used to further enrich the protein to a degree of purity sufficient for crystallisation (Volkel *et al.*, 1998). Another way is the purifiaction of a same affinity tag by different materials, first by a nickel(II) chelate and second with an anti-his antibody (Müller *et al.*, 1998). This may only be applicable in single cases. As a method needs to be standardised and materials kept on a simple and cheap basis, the use of a dual tag composed of metal binding sequences with different specificities should be anticipated. CASMAC is such a technique of consecutive columns with different immobilised metal ions (Porath and Hanssen, 1991). However, the cross reaction between the transition metals is high. The ideal dual tag would be composed of a nickel(II)-tag sequence and a iron(III)-tag sequence which have been found to be highly selective for their metal ligand in the presence of 20mM imidazole. The cascade of a nickel(II)-column followed by a iron(III)-column could be applied for CASMAC, leading to a homogeneous purification.

Novel affinity handles could be identified by the procedures performed in this work. The ideal parameters would be the use of an affinity material with a low backgound binding and a simple immobilised ligand which can also be introduced for the competitive elution of specifically retained phage particles. A more straightforward approach is the elevation of the binding constant of identified metal ion binding sequences by mutagenesis and affinity selection with phage-display, which may also result in more specificity for the affinity material. This customisation can be performed in general if large scale preparations of recombinant proteins with maximum affinity and selectivity using a defined affinity material are anticipated. Further performance could be achieved by the addition of functional groups like aromatic residues in proximity to the immobilised chelate on the support. Extention of the affinity tag with a random sequence displayed on a phage would lead to the identification of peptides with augmented interactions with the chromatography material, thereby enhancing the binding properties.

5 SUMMARY

The aim of this work has been the identification and characterisation of metal binding petides by phage-display. It was demonstrated that selected peptides were specific for both metal ion and chelating support material. Specificity for a metal ion can be roughly divided into two subclasses, namely transition metal ions Cu(II), Co(II), Zn(II), Ni(II) and hard Lewis acids Al(III) and Fe(III).

The former class was known to preferentially bind to histidine residues which could be confirmed by peptide sequences identified in this work. The highest affinity was found to be associated with four consecutive histidine residues, bracketted with hydrophobic amino acids and proline. Specificity for chelating support material correlates with the position of the binding motiv relative to N-terminus of the peptide and general hydrophobicity. This results in a preference for a central motif orientation and hydrophilic residues for IDA and an N-terminal motif orientation and more hydrophobic residues for INDIA-chelates.

Peptides binding to hard Lewis acids share properties of a marked positive net charge accompanied with histidine residues. Binding strength is dependent on a balanced distribution of these properties as displayed by the variant FeSZIV#1 which also showed a high affinity for Ni(II) in absence of competing imidazole. The chelating support has only a minor role with respect to affinity, resulting in a stronger binding to IDA.

Cross-reactivity of peptides with different classes of metal ions can be abolished completely with low molarities of imidazole. The peptides retained their affinity even under strong denaturing conditions and high ionic strength. These properties match those described for the well characterised hexahistidine-tag. This suggests that these novel tags could be a powerful alternative in those cases where classical approach is unsuccessful.

The use of the metal binding peptides for purification of fusion phage was demonstrated. A novel helper phage M13Co1 which carries such a metal chelate-binding tag was constructed which can be purified from or quantified in mixtures of other phage/phagemid.

Phage-display offers coupling of *in vivo* selection of variant molecules in terms of expression and replication with *in vitro* selection with respect to target binding. Since selective binding and good compatibility with host expression system are hallmarks of a

valuable affinity tag, phage display provides optimal features for the discovery of such sequences. The protocols and hypotheses established this work should be generally useful for identification of novel sequences specific for other so far uncharacterised matrices. It is conceivable that further applications for affinity tags can be found and will augment the value of these as tools in biochemistry and medicine in the future.

6 ZUSAMMENFASSUNG

Ziel dieser Arbeit war die Indentifizierung und Charakterisierung Metallchelat bindender Peptide mittels Phage-Display. Es konnte gezeigt werden, daß die selektierten Peptide sowohl für die Metallionen als auch für das Chelatmaterial spezifisch sind. Die Spezifität für das Metallion kann in zwei Klassen unterteilt werden: Übergangsmetallionen Cu(II), Co(II), Zn(II), Ni(II) und harte Lewissäuren Al(III) und Fe(III).

Die erste Klasse von Metallionen war dafür bereits bekannt, in erster Linie an Histidinresten zu binden, was auch durch die in der Arbeit identifizierten Sequenzen bestätigt werden konnte. Die höchste Bindungsaffinität wurde in Verbindung mit 4 aufeinander abfolgenden Histidinresten, umgeben von hydrophoben Seitengruppen und Prolin, ermittelt. Die Spezifität für das Trägermaterial wird durch die Position des Bindungsmotives im Verhälnis zum N-Terminus des Peptides bestimmt, welches im Falle vom IDA-Chelat zentral und für INDIA-Chelat N-terminal gelegen ist.

Harte Lewissäure bindende Peptide haben in ihrer Sequenz eine augeprägte positive Nettoladung sowie mehrere Histidinreste gemeinsam. Die Bindungsstärke hängt von der speziellen Verteilung von positiver Ladung und den Histidinresten ab, wie sich bei dem Klon FeSZIV#1 gezeigt hat, der allerdings neben Fe(III) auch Ni(II) in Abwesenheit von Imidazol binden kann. Das Trägermaterial spielt bei der Affinität nur eine untergeordnete Rolle. Generell wurde jedoch eine stärkere Bindung bei IDA erreicht.

Die Kreuzreaktivität zwischen den beiden Metallionen-Klassen kann durch Zugabe von geringen Mengen an Imidazol völlig unterbunden werden. Die Peptide behielten ihre Affinität sogar unter denaturierenden Bedingungen sowie bei hoher Ionenkonzentration. Diese Eigenschaften sind vergleichbar mit dem gut untersuchten Hexahistidin-Tag und legen daher die Verwendung der neuen Sequenzen als eine Alternative in den Fällen nahe, wo die bisherige Anwedung mißlingt.

Die Anwendbarkeit der Metallionen bindenden Peptide für die Anreicherung fusionierter Bakteriophagen konnte gezeigt werden. Ein neuer Helferphage, M13Co1 der eine solche Chelat-bindende Sequenz enthält, wurde konstruiert, mit dessen Hilfe dieser aus Mixturen von anderen Phagen oder Phagemiden getrennt oder quantifiziert werden kann.

Die Technik des Phage-Displays ermöglicht die Kopplung von *in vivo* Selektion von Molekülvarianten anhand von Expressions- und Replikationseffizienz in *E. coli* mit der

in vitro Selektion durch Ligandeninteraktion. Da selektive Bindung sowie Kompatibilität mit dem Expressionsystems des Organismus den Wert eines Affinitätstags ausmachen, ist Phage-Display das Mittel der Wahl zur Entdeckung ebendieser Sequenzen. Die in dieser Dissertation erarbeiteten Protokolle und Hypothesen sollten sich generell als hilfreich erweisen, um neue bisher uncharakterisierte Materialien bindende Sequenzen, zu identifizieren. Es ist vorstellbar, daß weitere Anwendungen für Affinitätstags gefunden werden, die den Wert dieser als Werkzeuge in Biochemie und Medizin erweitern werden.

7 MATERIALS AND METHODS

7.1 Materials

7.1.1 Chemicals

Unless stated otherwise, all chemicals used are of analytical grade and commercially available. All aqueous solutions were prepared from demineralised and autoclaved water.

7.1.2 Devices

Devices used for the experiments belong to the standard equipment of molecular biological laboratories. The listed devices and instruments are merely those which may influence the obtained results due to the specific measures and settings of the model applied.

Application	Model	Supplier
Centrifugation	Biofuge A	Heraeus Sepatech GmbH, Osterode, Germany
	Sigma 3K12	Sigma Laborzentrifugen GmbH, Osterode, Germany
	RC5C	DuPont de Nemours, Bad Homburg, Germany
Photometry	Multiskan MCC/340 MKII	Bartholomey Labortechnik, Alfter, Germany
	Spectronic Genesis 2	Milton Roy Company, Rochester, USA
	CytoFluor Multiwell Plate Reader Series 4000	PerSeptive Biosystems GmbH, Wiesbaden-Nordenstadt, Germany

	GeneQuant RNA/DNA Calculator	Pharmacia Biotech Europe GmbH, Freiburg, Germany
Agarose gel electrophoresis	Horizontal Gel Electrophoresis System Horizon 58	Bethesda Research Laboratories, Neu Isenberg, Germany
Polyacrylamide gel electrophoresis	Minigel Twin	Biometra GmbH, Göttingen, Germany
Western transfer	Semi-Dry-Blotting chamber	PHASE GmbH, Lübeck, Germany
DNA-sequencing	A.L.F.-DNA-Sequencer	Pharmacia Biotech Europe GmbH, Freiburg, Germany
Electroporation	Gene Pulser and Pulse Controller	Bio-Rad Laboratories GmbH, Munich, Germany
Shaker	Certomat U	B. Braun Biotech International GmbH, Melsungen, Germany
PCR	Peltier Thermal Cycler PTC-200	Biozym Diagnostik GmbH, Hess. Oldendorf, Germany

7.1.3 Computer software

DNA-analysis were performed with GenMon 4.3, GBFmbH, Brunswick, Germany, and Vector NTI Version 4.0, Informax Inc., North Bethesda, USA. Sequencing was controlled by A.L.F.-Manager 3.01, Pharmacia Biotech Europe GmbH, Freiburg, Germany.

7.1.4 Bacterial strains and bacteriophage

E. coli strain	Genotype/Phenotype	Reference
JM103	*endA, Δ(lac, pro), thi-1, strA, sbcB15, hsdR4, supE,* λ^- [F' *traD36,* $proA^+B^+$, $lacI^qZ\Delta M15$] (P1 lysogen)	Messing *et al.*, 1981
XL1-blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F' $proA^+B^+$, $lacI^qZ\Delta M15$ Tn*10* (Tet^r)]	Bullock *et al.*, 1987

Bacteriophage	Genotype/Phenotype	Reference
M13LP67	M13 phage, Ap^r, pIII-fusion with random 15mer	Devlin *et al.*, 1990
M13K07	M13 phage Km^r from Tn903, ori of p15A, packages phagemid DNA preferentially	Vieira and Messing, 1987
M13Co1	M13 phage, Km^r from Tn903, ori of p15A, pIII-fusion with His-tag, packages phagemid DNA preferentially	This work

7.1.5 Antibodies

Name	Comments	Provider
Anti-M13 antibody	HRP conjugate	Pharmacia Biotech Europe GmbH, Freiburg, Germany
Anti-pIII antibody 10C3	IgG_1, monoclonal mouse	Tesar et al. 1995
Goat anti-mouse-IgG+IgM (H+L)	HRP conjugate	Dianova GmbH, Hamburg, Germany

7.1.6 Antibiotics and growth media

Antibiotic stock solution	Composition
Ampicillin (50mg/ml)	1g Ampicillin Na-salt
	20ml 70% ethanol
Kanamycin (50mg/ml)	1g Kanamycin filtrated
	20ml dH_2O
Streptomycin (50mg/ml)	1g Streptomycin filtrated
	20ml dH_2O
Tetracyclin (20mg/ml)	400mg Tetracyclin
	50% dH_2O

Growth medium	Amount	ingredient
LB medium	10g	tryptone
	10g	yeast extract
	5g	NaCl
	ad 1l	H_2O
		autoclave
Agar stock solution (2x), autoclave	15g	agar
	ad 500ml	H_20
LB stock solution (2x), autoclave	10g	tryptone
	10g	yeast extract
	5g	NaCl

	ad 500ml	H_2O
M9-medium (1x), use sterile filter	100ml	M9-salt solution (10x)
	12.5ml	glucose (40% w/v)
	100µl	CaCl (1M)
	1ml	Mg SO_4
	1mM	$FeCl_3$
	100µl	thiamine
	Ad 1l	H_2O
M9-salt solution (10x), autoclave	74.1g	Na_2HPO_4*H2O
	30g	KH_2PO_4
	5g	NaCl
	10g	NH_4Cl
	Ad 1l	H_2O

Agar Plates:

For the preparation of agar plates, heat 500ml agar stock (2x) in the microwave oven until all the gel is dissolved. Mix heated agar stock with LB or M9 medium stock (2x) and let it cool down to about 50°C before adding antibiotics. Stir slightly before casting the argar plates.

Top Agar:

Take 1l of LB medium (1x) and add 6g of agar, autoclave. The heated solution is poured on top of already prepared agar plates, about 4ml each.

7.1.7 Buffers and solutions

Buffers

PBS (10x) autoclave	80g	NaCl
	2g	KCl
	14.3g	$Na_2HPO_4*2H_2O$
	2g	KH_2PO_4
	Ad 1l	H_2O
	Equilibrate to pH7	HCl
PBS-T (1x)	50ml	PBS (10x)
	450ml	H_2O
	250µl	Tween 20
Wash PBS (1x)	50ml	PBS (10x)
	50ml	5M NaCl stock solution
	400ml	H_2O
	250µl	Tween 20
400mM MOPS pH7.4 (10x) autoclave	41.9g	MOPS
	Equilibrate pH7.4	HCl
	Ad 500ml	H_2O
100mM MOPS pH7 (10x) autoclave	10.5g	MOPS
	Equilibrate pH7	HCl
	Ad 500ml	H_2O

Wash MOPS-T (1x)	50ml	MOPS pH7.4 (10x)
	20ml	5M NaCl stock solution
	250µl	Tween 20
TBS (10x) autoclave	61g	Tris
	80g	NaCl
	2g	KCl
	Ad 1l	H_2O
	Equilibrate pH7.4	HCl
TBS-T (1x)	50ml	TBS (10x)
	450ml	H_2O
	250µl	Tween 20
TA (25x)	121.1g	Tris
	18.6g	EDTA
	Equilibrate pH8	Acetic acid
	Ad 1l	H_2O
TBE (10x) filter	100g	Tris
	55.6g	Borate
	9.3g	EDTA
TE (1x) autoclave	121mg	Tris
	200µl	.5M EDTA
	Equilibrate pH8	HCl
	Ad 100ml	dH_2O

Tris 1M pH8 autoclave	12.1g	Tris
	Equilibrate pH8	HCl
	Ad 100ml	H_2O
Phoshate buffer 10% autoclave	10g	Na_3PO_4
	Ad 100ml	H_2O
Phosphate buffer pH4 autoclave	356mg	Na_2PO_4*H2O
	Equilibrate pH4	H_3PO_4
	Ad 100ml	H_2O
1M Lysine pH7.4 filter	14.6g/100ml H_2O	Lysine
1M Ethanolamine pH7.4 filter	6.1ml/100ml H_2O	Ethanolamine
1M Glycine buffer pH2.2 autoclave	7.5g/100ml H_2O	Glycine
Laemmli run buffer (8x)	560g	Tris
	120g	Glycine
	200ml	SDS(10%)
	80ml	.5M EDTA
	Ad 2.5l	H_2O
Upper Tris (10x)	15g	Tris
	20ml	SDS(10%)
	Equilibrate pH6.8	HCl
	Ad 100ml	H_2O
Lower Tris (10x)	91g	Tris
	20ml	SDS(10%)

	Equilibrate pH8.8	HCl
	Ad 500ml	H_2O
Transfer buffer (10x)	30.3g	Tris base
	144.1g	Glycine
	Ad 1l	H_2O
Transfer buffer (1x)	10ml	Transfer buffer (10x)
	20ml	Ethanol
	70ml	H_2O
Loading buffer SDS-PAGE (2x) store at – 20°C	121mg	Tris
	309mg	DTT
	400mg	SDS
	2ml	Glycerol
	20mg	Bromophenolblue
	Equilibrate pH6.8	HCl
	Ad 10ml	H_2O

Solutions

PEG/NaCl	100g	PEG 8000
	116.9g	NaCl
	475ml	H_2O
Stop solution	9.5ml	Formamide
	100µl	1M NaOH
	.5mg	bromphenolblue
Silane solution	2µl	3-Methacryloxypropyltrimethoxysilane
	500µl	Ethanol
	125µl	Acetic acid (10%)
APS store at –20°C	1g	Ammoniumpersulfate
	9.5ml	H_2O
Ethidiumbromide	50mg	Ethidiumbromide
	10ml	H_2O
NaN3 stock, protect from light	500mg	NaN_3
	20ml	H_2O
Coomassie stain, filter	1mg in 200ml ethanol	Coomassie G250
	1mg in 200ml H2O	Coomassie R250
	40ml	Acetic acid
Loading solution AGE (6x)	4g	Sucrose

	25mg	Bromphenolblue
	Ad 10ml	H_2O
DAB 2%, store at –20°C	200mg	diaminobenzidine
	10ml	H_2O

7.2 Methods

7.2.1 Cultivation of microorganisms

Bacterial strains were all cultured in erlenmeyer flasks with volumes ranging from 50ml to 2l which has been filled to a maximum of 1/3rd with LB supplemented with selective antibiotics. In absence of phage infection, cultures were incubated at 37°C. For phage production, a temperature of 28°C was used. The incubation was performed on shakers at 180rpm in air conditioned rooms set at the desired temperature.

7.2.2 Strain maintenance and glycerol stocks

For the short term maintenance of bacterial strains, single colonies were obtained by streaking out on a minimal medium agar plate from a glycerol stock aliquot. The minimal medium M9 containing the appropriate supplines should ensure the growth of cells harbouring the F' factor. This agar strain plate was kept for up to 2 weeks at 4°C before a new one was prepared from glycerol stock. A glycerol stock was prepared from 500µl overnight culture with the addition of 500µl glycerol in autoclaved cryo-tubes and frozen at –70°C for long term storage.

7.2.3 DNA methods

7.2.3.1 Preparation

In this work, only mini-preparations were necessary. In order to perform best results for sequencing and cloning, plasmid miniprep kits were used provided by GENOMED GmbH, Bad Oeynhausen, Germany (JETstar) and Quiagen GmbH, Hilden, Germany

(Quiagen-tip 20). The principle of these kits is based on the alkaline lysis of cells and DNA affinity purification on silicate materials.

7.2.3.2 Quantification

Quantification of DNA was performed by photometry measurements at 260 and 280nm wavelengths. A quartz cuvette filled with the appropriate dilution of the sample was placed into a photometer and measured at both wavelengths. The quotient of the absorptions 260:280 should be in the range of 1.8 to 2, otherwise impurities are present in the sample. The concentration of the DNA in µg/µl can be calculated as:

$$C = A_{260} \times \varepsilon_0^{-1} \times 1cm^{-1}$$

The values for the coefficient of absorption ε_0 are:

For double stranded DNA: .020 ($\mu l \times \mu g^{-1} \times cm^{-1}$)

For single stranded DNA: .025 ($\mu l \times \mu g^{-1} \times cm^{-1}$)

An alternative method for the estimation of DNA concentrations is the comparison of fluorescence of DNA fragments similar in size run in a parallel lane of the sample with known concentration in ethidiumbromide stained agarose gels.

7.2.3.3 Restriction

For the restriction of DNA, endonucleases obtained from New England Biolabs GmbH and Boehringer Mannheim GmbH (Mannheim, Germany) were applied. The reactions were performed in the buffers recommended by the provider. One µg of DNA was incubated with one unit of enzyme for 2 hours at 37°C. The volume was kept as small as possible, but not below 10 times the volume of the enzyme stock applied. Note that an amount of 5% glycerol originating from the stock can inhibit the enzyme reaction. The restriction was checked by agarose gel electrophoresis.

7.2.3.4 Agarose gel electrophoresis (AGE)

The running buffer in the gel chambers was TA (1x) and re-used only for one day. The agarose gels contained usually 1% agarose in TA (1x) buffer which is heated in the microwave oven in order to let the gel dissolve. The gels were cast in the gel trays with the appropriate combs inserted and sealed with scotch tape at the sides. When the gel became solid, the tray was transferred into the chamber and the comb withdrawn to produce the pockets for the loading of the samples. The λ-DNA digested with HindIII usually serve as a molecular weight standard to be run in parallel with the samples. The samples were mixed with the sample loading buffer for AGE and pipetted carefully into

the pockets, avoiding spillage of excessive sample. The electrophoresis was performed at a voltage of 60-100V and usually terminated when the bromphenolblue marker reached the end of the gel. The gel was the subjected to a staining in water containing 40µl of the ethidiumbromide stock solution per litre. After agitation for about 15 min, the gel was placed on an UV transilluminator and analysed at 302nm wavelength.

7.2.3.5 Elution of DNA-framgents from agarose gels

When the correct fragments were observed in the agarose gel after electrophoresis, excision of the band was performed by a clean scalpel and placed into an eppendorf tube. The elution from the gel was achieved by the use of the JetSorb Gel Extraction Kit supplied by GENOMED GmbH (Bad Oeynhausen, Germany). The weight of the gel fragment was determined to chose the right conditions suggested for the extraction. The result of the DNA elution was checked by another AGE.

7.2.3.6 Ligation

For the ligation reaction of DNA fragments, T4-ligase and the appropriate ligase buffer supplied by Gibco-BRL GmbH (Eggstein, Germany) was used. The fragments were added in approximately equimolar concentratoin to .01µg/µl, usually to reach a volume of 20µl. .5units of ligase was applied for µg of total DNA. The samples were incubated at 8°C for at least 4 hours. For transformation of the product, the samples were diluted 1:1 and incubated for 10 min at 65°C.

7.2.3.7 Sequencing

The sequencing of double stranded phage DNA was carried through by the so-called cycle sequencing procedure. This involves an asymmetric PCR with one fluoresceine-labelled primer and ddNTP, generating a single stranded DNA of different lengths which is amplified by this method. Advantage over the conventional sequencing is the decreased amount of template DNA necessary for the reaction and the more controlled conditions in a thermal cycler.

For the preparation of DNA, 3ml overnight cultures were lysed and purified by the miniprep kits described above. The elution of DNA was usually performed in 60µl TE buffer. An aliquot 10µl of the sample was then used for the reaction. The pipetting of all the solutions was performed on ice. The following buffers and solutions are required per reaction:

PD56 Primer sequence:

2µl	10x buffer	120mM	Tris

		40mM	$MgCl_2$
		150mM	$(NH_4)_2SO_4$
		Equilibrate pH 9.5	
1µl	Fluorescent primer	2pmol	FITC-DNA
4µl	dNTP	1mM	dATP
		1mM	dCTP
		1mM	7-deaza dGTP (Pharmacia GmbH, Freiburg, Germany)
		1mM	dTTP
.16µl	Taquenase/PPase	5 U Taq/ 1U PPase	Amersham Life Science, Inc.
3µl	dH_2O		
10µl	Sample DNA		

Aliquots of 5µl are pipetted into single wells of PCR-softstrips containing 2µl didesoxy-nucleotides to terminate the reaction in the thermal cycler.

5µM	ddATP
5µM	ddCTP
3.75µM	ddGTP
5µM	ddTTP

PCR setup

94°C initial denaturing	120sec	1 step
94°C denature	15sec	

52°C anneal	15sec	36 cycles
72°C extension	40sec	
72°C final extension	300sec	1 step
4°C stop	Until stop solution is added	

Add 4µl stop solution to each of the samples. Heat the samples for 2 minutes at 95°C and place them immediately on ice before loading on the sequencing gel.

Preparation of the sequencing slabs

The glass slabs of the ALF-sequencer were washed extensively with 10%SDS, dH_2O and ethanol (96%). The front plate was treated with silane solution in the area where the comb was inserted in order to ensure that the pockets stay intact when the comb is withdrawn. The assembly of the plates was done according to the producers instructions.

Contents of the sequencing gel (.5mm spacers, 6.6% polyacrylamide)

Urea (ALF-grade, Pharmacia)	33.6g
TBE (10x)	9.6ml
Acrylamide/bisacrylamide	17.6ml
(29%/1%, Pharmacia)	
dH_2O	Ad 80ml

The mixture was dissolved and de-gassed by vaacuum-filtration. The polymerisation was initiated with 65µl TEMED and 240µl APS. The gel was cast horizontally and left to polymerise for at least 2 hours after the insertion of the comb. The comb was removed directly before the gel run.

Gel run conditions

TBE (.6x) served as the run buffer. 1600V, 38mA, 45W, 50°C, 2.5sec interval of detection, 300min runtime were the settings entered in the ALF-Manager programme.

7.2.4 Identification of M13LP67 deletions

DNA samples obtained from single colonies and a control with known insert sequence were digested with ClaI and KpnI:

5µl DNA sample

1µl Buffer L (Boehringer GmbH, Mannheim, Germany)

.5µl ClaI (10u/µl)

.5µl KpnI (10u/µl)

3µl H_2O

The samples were incubated at 37°C for 2 hours and then subjected to AGE in an 1% agarose gel.. The smallest band of the control migrates at 981bp containing the N-terminal part of the pIII gene. Deletions in the region of the pIII library insert result in a loss of 69bp. This difference can easily visualised by the size dependent mobility in the AGE

7.2.5 Transformation of *E. coli*

7.2.5.1 Preparation of electro-competent cells

An 10ml LB overnight culture containing the appropriate antibiotics of the E. coli strain to be prepared is inoculated first. One percent of this culture was served as a starter culture for the 2x500ml media in 2l erlenmeyer flasks to be grown up to an OD_{600} of .6 at 37°C and 180rpm. Harvest of the cells was achieved by centrifuging aliquots of 250ml in a GS3 rotor, RC5C for 15min at 8000rpm and 4°C. Each pellet was resuspended in 250ml ice-cold dH_2O and centrifuged again. After resuspension with 125ml ice-cold dH_2O, two aliquots were pooled to be centrifuged again. The pellets were then resuspended in 10ml ice-cold 10% glycerol and pooled in GSA tubes. After the final centrifugation for 15min at 8000rpm and 4°C (GSA rotor, RC5C), the pellets resulting from 1l culture were resuspended with a total volume of 2ml 10% glycerol. All of these preparation steps were carried out at 4°C. The cell suspension is filled as 50µl aliquots into eppendorf tubes and shock frozen in liquid nitrogen. These aliquots were finally stored at –70°C.

7.2.5.2 Electroporation

For electroporation 50µl aliquots of electrocompetent cells were thawed slowly on ice. 1µl of the ligated sample corresponding to about .02µg DNA was applied to each of the

aliquots on ice and incubated for one minute. The suspension was transferred to previously chilled electroporation cuvettes with a 2mm gap (Electroporation Cuvettes Plus 2mm gap, BTX Inc., San Diego, USA) and subjected to the following conditions in a gene-pulser: 2.5kV, 25µF and 200Ω. One ml of LB supplemented with 20mM glucose was added to the cells right after the pulse. The cells were incubated for one hour in eppendorf tubes at 37°C before plating aliquots of 10 and 100µl on selective media. A mock transformation containing no added DNA served as a negative control.

7.2.6 Phage propagation

E. coli JM103 cultures were grown to an OD_{600} between .5 and .6 at 37°C before infecting them with bacteriophage. Usually, an aliquot of 1ml culture containing 10^8 cells was infected with 100µl phage eluate from the panning experiment. The cells were incubated without agitation for 25min at 37°C. The infected culture was then transferred to a 50ml LB containing 150µg ampicillin/ml and incubated overnight at 28°C and 180rpm to allow phage production.

7.2.7 Phage preparations

7.2.7.1 PEG/NaCl precipitation

The 50ml overnight cultures producing phage were centrifuged in GSA tubes at 8000rpm for 15min (GSA, RC5C). The supernatants were transferred into new GSA centrifuge tubes containing 7.5ml PEG/NaCl solution. The precipitation of the phage particles was achieved by incubation for at least 2 hours at 4°C. The particles were then pelleted by centrifugation at 10000 rpm for 45min at 4°C. The phage were resuspended by the addition of 500µl 40mM MOPS pH7.4 for the hard Lewis acid metal and 500µl PBS for the transition metal ion selections and transferred to eppendorf tubes. The phage suspensions were cleared of debris by centrifugation in an Heraeus table centrifuge for 10min at 13000rpm. The supernatants were transferred into new eppendorf tubes and finally sterilised by the addition of NaN_3 to .02% and stored at 4°C.

7.2.7.2 IMAC affinity purification of bacteriophage

Chelating Sepharose FF

Chelating Sepharose FF was incubated with 10mM $CoCl_2$ resulting in a salmon red colouring of the sepharose. After 10 minutes the supernatant was removed and new $CoCl_2$ solution was added. This was repeated until saturation in the colour was observed. Then the sepharose was rinsed several times with distilled water and finally

resuspended in 20% ethanol for storage at 4°C. Before use, the sepharose was rinsed with distilled water.

50 ml of a CoSZIV#7 LB overnight culture centrifuged and the supernatant was stored in the refrigerator at 4°C. 10ml of this supernatant were adjusted by addition of 1ml 10xPBS .5% Tween 20. The sample was put on a rocker and agitated mildly for 1.5 hours. A sterile 1ml disposable pipette tips were prepared with a small glass wool plug at the narrow end and placed in a rack. Then, the sample was loaded on the tip successively allowing the solution to pass through. When the whole 11ml passed through, the sepharose retained on the tip was washed with 1ml of wash-PBS containing 20mM imidazole. Elution was initiated by the addition of 100µl .5M imidazole in PBS. The remaining solution was removed from the tip by applying mild pressure with a 1ml pipette.

Talon Metal Affinity Resin

100µl of the above overnight culture supernatant was equilibrated with of 400µl PBS .5M NaCl .05% Tween 20 prior to addition of 20µl of Talon resin. The sample was mildly agitated for about 2 hours and loaded on a 1ml disposable pipette tip plugged with glass wool. The resin was the washed with 1ml of wash-PBS. Elution was performed with 100µl of .5M imidazole pH7.

7.2.8 Titre estimation of phage

7.2.8.1 cfu-assay

Dilutions were performed in microtitre plates in order to estimate the titre of several samples at a time. All wells were filled with 100µl dH_2O and 10µl of the samples were transferred into the first row. After a brief mixing with the multi-channel pipette, 10µl were transferred into from one row to the next, until the desired dilution factor was reached. The pipette tips were changed after each transfer to ensure a correct dilution. The infection is initiated by addition of 100µl E. coli culture at OD600 = .6 and incubation for 20min at 37°C. Aliquots of 20µl from each well were pipetted carefully on previously air-dried and marked agar plates with 150µg/ml ampicillin for M13LP67 or 100µg/ml kanamycin for M13K07. These plates were incubated overnight at 28°C to allow colony formation of infected cells. The colonies formed on a dilution factor spot were counted. The titre was calculated by the multiplication of colonies formed with the dilution factor.

7.2.8.2 pfu-assay

About 4ml of top agar/plate was melted in the microwave oven. The solution was cooled down to 41°C before 100µl of E. coli log-culture was added and the gel was cast on pre-warmed LB plates (37°C). The top agar plates were incubated for 30min at 37°C before infection with phage. Like the cfu-assay, the dilution of samples were performed identically on a microtitre plate. Aliquots of 10µl per well were pipetted on marked spots of the top-agar plate. The plates were then incubated overnight at 37°C to allow plaque formation on the bacterial lawn. The titre was calculated by multiplication of counted plaques on a spot by the dilution factor.

7.2.9 Preparation of chromatography materials

7.2.9.1 SpinZyme

The affinity membrane comes readily charged with iron(III). As the producer recommends an incubation with 15% formic acid for removal, two washing steps with 500µl were performed, followed by two additional washing steps with .5M EDTA. Let the solutions incubate at room temperature for about an hour at each washing step to ensure the complete removal of iron(III). The solutions were spun down in a table centrifuge at 4000 rpm. In order to remove residual EDTA, two washing steps with dH_2O were carried through. The membrane was charged with the new metal salt, usually at 50mM with chloride a the counter ion in MOPS pH 7.4, by two incubations of 500µl of this solution. Finally, the excessive salt was washed away with two washing steps of 500µl dH_2O.

7.2.9.2 ReactiBind

The microtitre-plates are shipped with nickel(II)-charged INDIA immobilised to the bottom of the wells. To remove the nickel ions from the material, the same conditions as for SpinZyme were chosen. Only the volumes are adjusted to 400µl for each solution. The plate is slapped out in a sink and then thoroughly on a paper towel to remove any residual solution from the wells.

7.2.9.3 NTA-sepharose

A volume of 200µl Ni(II)-NTA resin was washed once with dH_2O and then resuspended with 500µl of 50mM EDTA for 20min at room temperature. This was repeated once and then washed five times with 500µl dH_2O. The resin was stored at 4°C until further use. Just before use, the resin was charged with iron(III). 500µl 10mM $FeCl_2$ solution was added to the resin and incubated for 20min at RT. This incubation was repeated once before removing the excessive iron from the material. Wash again five times with 500µl

dH_2O. Do not use any neutral or alkaline buffer which could cause the unbound iron(III) precipitate as oxides.

7.2.10 Purification of pIII fusions

7.2.10.1 Cu(II) SpinZyme

Varying amounts of urea were added to different samples containing some $5x10^{10}$ CoSZIV#5 to reach concentrations of 6.4M, 3.2M and 1.7M urea in 100µl respectively. These were kept at 4°C over night for denaturation.

Step	Volume	Buffer
Loading	100µl	Samples with urea
Washing	500µl	Wash-PBS-T 20mM imidazole 2M urea
	500µl	dH_2O
Elution	50µl	pH4 phosphate buffer
Neutralisation	12µl	10% phosphate

7.2.10.2 Chelating Sepharose Fast Flow

For the purification of the pIII protein, the nickel(II) charged Chelating Sepharose FF was prepared as described for cobalt(II). Samples NiSZIV#18 and NiRBIV#1 both containing about $3x10^{11}$ phage particles were used. End concentrations of 6.4M, 3.2M and 1.7M urea in 100µl were tested. Samples were kept over night at 4°C for denaturation. To each sample 10µl of equilibrate Ni(II)-Chelating Sepharose FF was added. Those were incubated for over 2 hours at room temperature to allow the proteins to interact. The samples were loaded on 1ml disposable pipette tip plugged with glass wool.

Step	Volume	Buffer
Loading	110µl	Samples with urea and 10µl resin
Washing	1000µl	Wash-PBS-T 20mM imidazole 2M urea
Elution	50µl	.5M imidazole pH7.4

7.2.10.3 Talon Affinity Resin

100µl of prepared CoSZIV#7 phage corresponding to $2.2x10^{11}$ cfu were denatured by the addition of 900µl of 8M urea resulting in a total concentration of 7.2M urea. The sample was incubated in presence of 20µl of Talon resin for over 2 hours at room temperature before being loaded on a 1ml disposable pipette tip plugged with glass wool.

Step	Volume	Buffer
Loading	1020µl	Samples with 7.2M urea and 20µl resin
Washing	1000µl	PBS 4M urea
Elution	50µl	.5M imidazole pH7.4
	50µl	.5M imidazole pH7.4 + 1%SDS

7.2.10.4 Ni(II)-NTA agarose

Urea variation protocol

100µl samples containing $2.2x10^{11}$ prepared CoSZIV#7 phage and 2, 4 and 6M urea were heated for 20 minutes at 65°C. Then 20µl Ni(II)-NTA was added and the volume set to 200µl by adding the appropriate amount of urea in PBS to keep the concentration of urea constant. These were kept at 4°C over night to be loaded on a 1ml disposable pipette tip with a glass wool plug on the next morning.

Step	Volume	Buffer
Loading	1020µl	Samples with 7.2M urea and 20µl resin
Washing	1000µl	PBS 4M urea
Elution	50µl	.5M imidazole pH7.4
	50µl	.5M imidazole pH7.4 + 1%SDS

7.2.10.5 Fe(III)-NTA agarose

The purification was started by incubation of $2.4x10^{10}$ phage separately in varying concentrations of urea (2M, 4M and 6M) in 1ml wash-MOPS .5M NaCl pH 7.4. The samples were heated for 20 min at 65°C. After cooling, 20µl of the Fe(III)-NTA resin

were added and incubated for 1 hour at RT with gentle agitation. The samples were loaded on 1ml disposable pipette tips plugged with glass wool.

Step	Volume	Buffer
Loading	1020µl	Samples with urea and 20µl resin
Washing	1000µl	Wash-MOPS-T .5M NaCl pH 7.4
Elution	100µl	.05M EDTA pH8
	50µl	.05M EDTA pH8 + 1%SDS

7.2.10.6 Comparison of Fe(III) and Ni(II)-NTA agarose

7.2.11 Selection procedures

7.2.11.1 Transition metal ions

Before the phage are loaded on the affinity material, the surfaces have to be blocked with protein to reduce background binding. This is achieved by the incubation with BSA for about one hour at RT. The phage are subsequently added with BSA to the material and incubated either for 2 hours at RT, or over night at 4°C. The initial round was performed with usually 100µl of phage preparation, containing about 10^{11}cfu. Later rounds were started with 50µl of phage preparation. The same amount of blocking buffer was added to reach a final concentration of 1.5%BSA.

	SpinZyme	ReactiBind	Buffer
Blocking	500µl	400µl	3% BSA in wash-PBS-T
Incubation	100-200µl	100-200µl	1.5%BSA wash-PBS-T+phage

For all the different transition metals and titanium(IV), wash-PBS-T was used as a washing and incubation buffer. Incubation times between the washing steps were at least 10min, each.

Selection round	Washing steps SpinZyme	Washing Steps ReactiBind	Buffer
I	500µl	400µl	3% BSA in wash-PBS-T
	5x500µl	3x400µl	Wash-PBS-T
II	500µl	400µl	3% BSA in wash-PBS-T
	5x500µl	3x400µl	Wash-PBS-T+20mM imidazole
III	500µl	400µl	3% BSA in wash-PBS-T
	10x500µl	5x400µl	Wash-PBS-T+20mM imidazole
IV	500µl	400µl	3% BSA in wash-PBS-T
	10x500µl	5x400µl	Wash-PBS-T+20mM imidazole

The elution from the metal chelate was achieved by the incubation with 100µl .5M imidazole adjusted to pH7.4 for 20 minutes at RT. Titanium(IV) binding variants were eluted with 100µl pH4 phosphate buffer and subsequently neutralised with a 10% phosphate buffer. The eluted phage population was then subjected to propagation in *E. coli* JM103.

7.2.11.2 Hard Lewis acid ions

The incubation times were kept identical to those of the selections on transition metal ions.

Aluminium(III) SpinZyme

	SpinZyme	Buffer
Blocking	500µl	3%BSA in wash-MOPS-T pH7
Incubation	100-200µl	1.5%BSA in wash-MOPS-T pH7+phage

Selection round	Washing steps	SpinZyme	Buffer
I		500µl	3%BSA in wash-MOPS-T pH7
		5x500µl	Wash-MOPS-T pH7
II		500µl	3%BSA in wash-MOPS-T pH7
		5x500µl	Wash-MOPS-T pH7
III		500µl	3%BSA in wash-MOPS-T pH7
		10x500µl	Wash-MOPS-T pH7
IV		500µl	3%BSA in wash-MOPS-T pH7
		10x500µl	Wash-MOPS-T pH7

Other hard Lewis acid ion selections were performed under the following conditions.

	SpinZyme	ReactiBind	Buffer
Blocking	500µl	400µl	3%BSA in wash-MOPS-T pH7.4
Incubation	100-200µl	100-200µl	1.5%BSA in wash-MOPS-T pH7.4+phage

Selection round	Washing steps SpinZyme	Washing Steps ReactiBind	Buffer
I	500µl	400µl	3%BSA in wash-MOPS-T pH7.4
	5x500µl	3x400µl	wash-MOPS-T pH7.4
II	500µl	400µl	3%BSA in wash-MOPS-T pH7.4
	5x500µl	3x400µl	wash-MOPS-T pH7.4
III	500µl	400µl	3%BSA in wash-MOPS-T pH7.4
	10x500µl	5x400µl	wash-MOPS-T pH7.4
IV	500µl	400µl	3%BSA in wash-MOPS-T pH7.4
	10x500µl	5x400µl	wash-MOPS-T pH7.4

Elution was achieved by the addition of 100µl 50mM EDTA pH8. The phage were removed from the material after 20min incubation at RT. These phage populations were propagated in *E. coli* JM103.

7.2.11.3 Fast lane panning

The affinity material chosen for this panning experiment was nickel(II) on ReactiBind. The first round was performed identical to the transition metal ion selections described above. In the following rounds II-IV, the phage preparation by PEG/NaCl was omitted and the phage contained in the supernatant of the overnight cultures were loaded directly on the material.

	ReactiBind	Buffer
Blocking	400µl	3% BSA in wash-PBS-T
Incubation	400µl	Culture supernatant

Due to the different elution modes, an additional washing step with dH_2O was performed.

Selection round	Washing steps	Buffer
I	400µl	3% BSA in wash-PBS
	3x400µl	Wash-PBS
	400µl	H_2O
II	400µl	3% BSA in wash-PBS
	3x400µl	Wash-PBS+20mM imidazole
	400µl	H_2O
III	400µl	3% BSA in wash-PBS
	5x400µl	Wash-PBS+20mM imidazole
	400µl	H_2O
IV	400µl	3% BSA in wash-PBS
	5x400µl	Wash-PBS+20mM imidazole
	400µl	H_2O

Elution was accomplished by to different approaches. FLA samples were eluted by a phosphate buffer at pH4 and FLB sample by the conventional imidazole elution buffer, each for 15 minutes at room temperature.

7.2.11.4 Cross-reactivity assays

Transition metal ions

Step	SpinZyme	ReactiBind	Buffer
Blocking	500µl	400µl	3% BSA in wash-PBS-T
Incubation	100µl	100µl	3%BSA wash-PBS-T + 10µl phage
Washing	5x500µl	3x400µl	wash-PBS-T
Elution	100µl	100µl	.5M imidazole pH7.4

Hard Lewis acids

Step	SpinZyme	ReactiBind	Buffer
Blocking	500µl	400µl	3% BSA in wash-MOPS-T
Incubation	100µl	100µl	3% BSA in wash-MOPS-T + 10µl phage
Washing	500µl	400µl	3% BSA in wash-MOPS-T pH7.4
	4x500µl	2x400µl	wash-MOPS-T pH7.4
Elution	100µl	100µl	50mM EDTA pH8

7.2.12 Protein analysis

7.2.12.1 Discontinuous polyacryamide gel electrophoresis (Laemmli, 1970)

The glass plates for the slab gel were cleaned with 10%SDS, water and ethanol before use.

SDS-Polyacrylamide gel components (Biometra Minigel, 12.5%)

Separating gel:

4.1ml	Lower Tris
3.2ml	dH_2O
2.5ml	29%acrylamide/1%bisacrylamide
8µl	TEMED
30µl	APS

Stacking gel:

2ml	Upper Tris
4.2ml	dH_2O
700µl	29%acrylamide/1%bisacrylamide
10µl	TEMED
35µl	APS

The gel was cast vertically with the separating gel first, leaving appropriate space for the stacking gel and the comb. The top was covered with ethanol and the gel was left to polymerise for about 30min at RT. Before adding the stacking gel, the ethanol was decanted. Finally, the comb was inserted and incubation was repeated for the polymerisation of the stacking gel. The electrophoresis chamber was filled with Laemmli run buffer (1x) when the gel was inserted. Residual air bubbles between the buffer and the gel were removed by a filled syringe. Samples were mixed 1:1 with SDS-PAGE loading buffer and heated for 10min at 95°C. 100V were applied for the run after the samples were loaded into the pockets. The run was finished when the bromphenol blue reached the end of the gel.

7.2.12.2 Silver staining of proteins

Due to the superior results obtained compared to manual preparation of staining solutions, silver staining was performed with the Daiichi silver stain kit II, Daiichi Fine Chemicals Corp. Tokyo, Japan.

7.2.12.3 Coomassie staining

Two different methods were used for the coomassie staining. The time saving method follows the incubation of an SDS-gel for 10min at 65°C in coomassie stain on a rocker. However, mostly the gels were left to staining in coomassie overnight on a rocker at room temperature. The coomassie staining solution was recollected through a paper filter into the storage bottle. De-staining was achieved by placing the stained gel into a plastic container with dH_2O and heat the water in a microwave oven shortly before the boiling point. The container the gel was then placed on a rocker for 5min before the water was replaced with fresh one. The process of heating, incubation and water change was repeated until the bands of the proteins became well defined above the background for analysis.

7.2.12.4 Western blot

Transfer to nitrocellulose

Six sheets of Whatman 3MM paper (Whatman International Ltd., Maidstone, England) and one nitrocellulose membrane (type HAHY, .45µm pore size, Millipore GmbH, Eschborn, Germany) were cut to the size of the SDS-gel. Three whatman papers soaked with transfer buffer (1x) were placed on the anode of the transfer chamber. The nitrocellulose membrane was also soaked in transfer buffer and placed above the stack of Whatman paper. The SDS-gel was incubated for 20min in transfer buffer and put on top of the nitrocellulose membrane. Finally, tree layers of Whatman paper soaked with transfer buffer was put on the stack. A 25ml glass pipette was rolled with mild pressure on the stack to remove residual air bubbles between the sheets. The cathode was put in place on the chamber and 100mA were applied for two hours.

Immunoblot

The membrane from the western transfer was blocked for at least 1 hour in 3%BSA in PBS-T. For pIII detection, the anti-pIII antibody was added in a dilution of 1:200 in PBS-T. The membrane was incubated for at least 2 hours. Two washing steps were performed with PBS-T for about 10min each. The anti-mouse antibody was added in a dilution of 1:5000 to PBS-T and incubated for at least one hour. Finally, the membrane

was washed 3 times before the addition of the freshly prepared peroxidase substrate solution.

peroxidase substrate solution:

20ml PBS-T

200µl Diaminobenzidine (2%)

200µl $NiCl_2$ (10%)

20µl H_2O_2

The development of the stain was stopped by rinsing the membrane extensively with water when bands of the detected proteins became apparent. The membrane was dried for storage and documentation. As the anti-M13 antibody is conjugated with horse radish peroxidase, a single incubation with this antibody in a dilution of 1:1000 was sufficient for the detection of pVIII by the staining solution.

7.2.12.5 ELISA

Phage-ELISA

Reacti-Bind microtitre plates (Pierce,) were used for the immobilisation of phage to the chelated nickel(II). The plate was pre-blocked with 3%BSA in PBS .05% Tween 20. The prepared phage were added in a volume of 10µl in 100µl PBS Tween 20 per well and incubated for two hours. The plate was washed four times with PBS-Tween 20 and the incubated with 100µl per well of anti-M13-Ab in PBS for additional two hours. Detection was performed after three washing steps with PBS-Tween 20 with freshly prepared peroxidase substrate solution. The absorbance at 475nm was scanned 5 min after the addition of the substrate and again after 10 min when the reaction was stopped with sulphuric acid.

Peroxidase substrate solution

4.8mg o-phenylenediamine

6ml dH_2O

3.1ml .2M Na_2HPO_4

2.9ml .2M citric acid

1.5µl H_2O_2 (30%)

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9 APPENDIX

9.1 Abbreviations

AGE	agarose gel-electrophoresis
APS	ammonium persulfate
BSA	bovine serum albumin
DAB	3,3'-diaminobenzidine
dH_2O	distilled water
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetate
ELISA	enzyme-linked immunosorbent assay
IDA	iminodiacetic acid
MOPS	morpholinopropane sulfonic acid
NTA	nitrilotriacetic acid
PAGE	polyacrylamide gel-electrophoresis
PEG	polyethylene glycol
PCR	polymerase chain reaction
SDS	sodium dodecylsulfate
TEMED	N,N,N'N'tetramethylethylenediamine
Tris	Tris(hydroxymethyl)-aminomethane
Tween 20	polyoxyethylenesorbitan monolaurate

9.2 Amino acid codes

Amino acid	Three letter code	One letter code	Properties	Side-chain pKa value
Alanin	Ala	A	Aliphatic	-
Arginine	Arg	R	Basic	12.4
Asparagine	Asn	N	Amide	-
Apartate	Asp	D	Acidic	4.4
Cysteine	Cys	C	Sulfur	8.5
Glutamine	Gln	Q	Amide	-
Glutamate	Glu	E	Acidic	4.4
Glycin	Gly	G	Aliphatic	-
Histidine	His	H	Basic	6.5
Isoleucine	Ile	I	Aliphatic	-
Leucine	Leu	L	Aliphatic	-
Lysine	Lys	K	Basic	10.5
Methionine	Met	M	Sulfur aromatic	-
Phenylalanin	Phe	F	Aromatic	-
Proline	Pro	P	Aliphatic	-
Serine	Ser	S	Hydrophilic	-
Threonine	Thr	T	Hydrophilic	-
Tryptophan	Trp	W	Aromatic	-
Tyrosine	Tyr	Y	Aromatic	10,1
Valine	Val	V	Aliphatic	-

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